ESTABLISHMENT OF A GENETIC DATABASE AND MOLECULAR METHODS FOR THE IDENTIFICATION OF FISH SPECIES AVAILBLE ON THE SOUTH AFRICAN MARKET

DONNA-MAREÈ CAWTHORN

Dissertation presented for the degree of

DOCTOR OF PHILOSOPHY (FOOD SCIENCE)

in the Faculty of AgriSciences at Stellenbosch University

Promotor: Prof. R.C. Witthuhn

Co-promotor: Dr. H.A. Steinman

December 2011

DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

08 November 2011

Donna-Mareè Cawthorn

Date

ABSTRACT

Consumers have the right to accurate information on the fish products they purchase to enable them to make educated seafood selections that will not endanger their own wellbeing or the wellbeing of the environment. Unfortunately, marine resource scarcity, financial incentives and inadequate or poorly enforced regulations have all promoted the mislabelling of fish species on global markets, the results of which may hold economic, conservation and health consequences. The primary aims of this study were to determine the most commonly available fish species on the South African market, to establish and compare DNA-based methods for the unambiguous identification of these species and to utilise the most applicable methods to evaluate the extent of mislabelling on the local fisheries market. The results from surveys of n = 215 restaurants and n = 200 retail outlets in four South African provinces (Western Cape, Kwa-Zulu Natal, Eastern Cape and Gauteng) indicated that 34 and 70 nominal fish types were available in restaurants and retail outlets, respectively, the most common of which were kingklip, salmon and hake. Over 30% of the fish species being sold were of conservation concern, while several outlets marketed specially-protected, illegal-to-sell species in South Africa. Fish purveyors were poorly equipped to provide information on the identity, origin, production method (farmed/wild) and sustainability of the fish they were selling and the labelling of many packaged fish products was in contravention with South African regulations. Data were published for the first time comparing the efficiency of five methods (urea-SDS-proteinase K, phenol-chloroform, salt extraction, SureFood® PREP kit and Wizard® Genomic DNA Purification kit) for the extraction of DNA from the muscle tissue of fish species available in South Africa. The SureFood® kit was identified as the most suitable method for DNA extraction from fish muscle, extracting significantly ($P < 0.05$) higher DNA yields than all other methods evaluated and being simple and safe to use. A comprehensive reference library of genetic information was compiled for the first time that contains sufficient DNA sequence data from different mitochondrial DNA loci (16S ribosomal RNA (rRNA), 12S rRNA and cytochrome *c* oxidase I (COI) genes, as well as the control region) to allow the explicit identification of 53 fish species in South Africa. Although 16S and 12S rRNA gene sequencing allowed the identification of most fish to the genus level, the discrimination of closely-related, congeneric species was problematic when based on these gene regions. Conversely, the vast majority (98%) of fish examined could be readily differentiated by their COI sequences, with only members of the genus *Thunnus* requiring supplementary control region sequencing for species confirmation. Lastly, sequencing of the COI region was used to show that 9% of fish samples collected from local seafood wholesalers and 31% of samples from retail outlets were mislabelled. This study has established that fish mislabelling is a reality on the South African market and that DNA-based methods should be applied by both industry and regulatory bodies to deter illegal activities and to promote transparency on the domestic fisheries market.

UITTREKSEL

Verbruikers het die reg tot akkurate informasie rakende die visprodukte wat hulle aankoop. Hierdie inligting sal hulle bemagtig om ingeligte seekos keuses te maak wat voordelig sal wees vir beide die verbruiker se eie, sowel as die omgewing, se voortbestaan. Ongelukkig het 'n gebrek aan seelewebronne, geldelike aansporings en onvanpaste of swak geïmplimenteerde regulasies gelei tot die verkeerde etikettering van visspesies op die wêreldmarkte. Dit mag ekonomiese-, bewarings- en gesondheidsgevolge inhou. Die primêre doelwitte van hierdie studie was om te bepaal watter visspesies die algemeenste beskikbaar is in die Suid-Afrikaanse mark, om DNS-gebaseerde metodes vir die duidelike identifisering van hierdie spesies te vind en te vergelyk, en om die mees gepaste metodes te gebruik om die omvang van verkeerde etikettering in die plaaslike vismarkte te evalueer. Die resultate van opnames van n = 215 restaurante en n = 200 winkels in vier Suid-Afrikaanse provinsies (Wes-Kaap, Kwa-Zulu Natal, Oos-Kaap en Gauteng) het gewys dat 34 en 70 nominale visspesies in onderskeidelik restaurante en kleinhandelaars beskikbaar was. Koningklip, salm en stokvis was die mees algemene spesies. Meer as 30% van die visspesies wat te koop was is van bewaringsbelang, terwyl verskeie winkels spesiaal-beskermde, onwettig-om-te-verkoop spesies in Suid-Afrika bemark het. Visverkopers was swak bemagtig om informasie oor die identiteit, oorsprong, produksiemetode (teel/wild) en volhoubaarheid van die vis wat hulle verkoop het te kon gee. Verder was die etikettering van baie verpakte visprodukte in stryd met Suid-Afrikaanse regulasies. Vir die eerste keer is data gepubliseer wat vyf metodes (ureum-SDS-proteïenase K, fenolchloroform, sout-ekstraksie, SureFood® PREP stel en Wizard® Genomic DNS suiwering stel) vergelyk in hul doeltreffendheid om DNS vanuit die spierweefsel van visspesies wat in Suid-Afrika beskikbaar is te ekstraheer. Die SureFood® stel is as die mees geskikte metode vir DNS ekstraksie vanuit visweefsel geïdentifiseer aangesien die DNS opbrengs betekenisvol ($P < 0.05$) hoër was met hierdie metode, en dit ook 'n eenvoudige en veilige metode is om te gebruik. 'n Omvattende verwysingsbiblioteek van genetiese informasie wat voldoende DNS volgordebepalingsdata van verskillende mitokondriale DNS lokusse (16S ribosomale RNS (rRNS), 12S rRNS en sitochroom *c* oksidase I (COI) gene, sowel as die kontrolegebiede) bevat, is vir die eerste keer opgestel om die besliste identifisering van 53 visspesies in Suid-Afrika toe te laat. Alhoewel 16S en 12S rRNS geenvolgordebepaling die identifisering van meeste visse op genusvlak toegelaat het, was die diskriminasie van naby-verwante, gelyksoorting spesies problematies

wanneer hierdie geengebiede gebruik is. Die oorgrote meerderheid (98%) vis wat ondersoek is geredelik onderskei op grond van hul COI volgordebepalings, met slegs lede van die genus *Thunnus* wat addisionele kontrolegebied volgordebepaling vir spesies bevestiging vereis het. Laastens, is volgordebepaling van die COI-gebied gebruik om te wys dat 9% van die vismonsters van plaaslike seekosgroothandelaars en 31% van die monsters van kleinhandelaars verkeerd geëtiketteer is. Hierdie studie het bevestig dat die verkeerde etikettering van vis in Suid-Afrika 'n realiteit is, en dat DNS-gebaseerde metodes gebruik moet word deur die industrie sowel as die regulerende liggame om onwettige aktiwiteite teen te werk en om deursigtigheid in plaaslike vismarkte te bevorder.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following persons and institutions for their valuable contributions to the successful completion of this research:

My promotor Prof. R.C. Witthuhn and co-promotor Dr. Harris Steinman for their continual guidance, knowledge and positive criticism during this study;

The University of Stellenbosch (Merit Bursary, 2008 - 2010), Ernst and Ethel Eriksen Trust (2008 and 2010) and South African Association of Food Science and Technology (SAAFoST) (Koeppen Memorial Scholarship, 2008) for financial assistance. The National Research Foundation (NRF) (Scarce Skills Bursary, 2008 - 2010) are hereby acknowledged for financial support (any opinion, findings and conclusions or recommendations expressed in this material are those of the author(s) and therefore the NRF does not accept any liability in regard thereto);

Graham Brill (Irvin & Johnson), Mark Goodman and Tracey Fairweather (Department of Agriculture Forestry and Fisheries), Gregg Stubbs and Johan Biggs (Three Streams Smokehouse), Damian Trigg (SupaPackers), Wiehan Visagie (Aquaculture Division of the Department of Genetics, Stellenbosch University), Roelof Brink (Fish Online) and Zibi Ferreira (Blue Marine) for the provision of fish samples and assistance with morphological identifications;

John Duncan (South African Sustainable Seafood Initiative), Jaco Barendse (RealFish), Martin Purves (Marine Stewardship Council) and Pieter Truter (National Regulator for Compulsory Specifications) for advice and expert guidance;

Staff and fellow post-graduate students at the Department of Food Science for support and friendship;

Sean Falconer for editorial assistance;

My parents, family and Riaan de Kock for their love, patience and continuous encouragement; and

My Heavenly Father for giving me the aptitude, motivation and strength to succeed.

For that which is common to the greatest number has the least care bestowed upon it.

— *Aristotle (384-322 BC)*

Dedicated to the ocean and its exhaustible fish stocks

CONTENTS

Language and style used in this dissertation are in accordance with the requirements of the *International Journal of Food Science and Technology*. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CHAPTER 1

INTRODUCTION

In 1883, the British biologist Thomas Huxley declared in his inaugural address to the International Fisheries Exhibition in London that ‘probably all the great sea fisheries are inexhaustible; that is to say that nothing we do seriously affects the number of fish’ (Jennings *et al*., 2001). Huxley was not to know that, just a few decades later, the demand for fish from an ever-increasing human population would spur a massive expansion of fishing fleets and fishing efforts, exceeding the ocean’s ecological limits and exhausting the inexhaustible (Delgado *et al*., 2003; Anyanova, 2008). The world today is facing a fisheries crisis (Clark, 2006). The global fishing fleet is estimated to be more than twice the size that the ocean can sustainably support, meaning that commercial fishing is removing fish faster than nature’s ability to replenish them (Porter, 1998). Harmful government subsidies have promoted the modernisation of fishing vessels and have only encouraged overfishing, while unfair fisheries partnership agreements have permitted foreign fleets to overfish the waters of many developing countries (Sumaila *et al*., 2007; Anyanova, 2008). Advancements in fishing gear have not only allowed greater numbers of target fish species to be harvested, but the unselective nature of these methods generate enormous amounts of ‘bycatch’ of non-target species (*ca.* 40% of the total fish capture), which is then either discarded or converted to animal feed (Davies *et al*., 2009).

The responses of the international community to the overfishing crisis have included the institution of numerous constraints on fisheries (rights allocations, annual harvest quotas, seasonal or area closures and fishing gear restrictions), a greater reliance on aquaculture to sustain the human demand for fish products, as well as the initiation of numerous seafood awareness campaigns that attempt to shift consumer purchasing behaviour towards more sustainable fish species (Jacquet & Pauly, 2007; FAO, 2009). Nonetheless, these endeavors have had limited success in reversing marine fisheries declines and data released by the United Nations Food and Agriculture Organization (FAO) indicate that up to 75% of global fish stocks are currently depleted, overexploited or at their maximum sustainable yields (FAO, 2009). With fish stocks dwindling and fishing quotas becoming increasingly stringent, the incidence of illegal, unreported and unregulated (IUU) fishing has soared in virtually all regions of the globe, the magnitude of which is estimated to account for 30% of total global catches and up to $23 billion per annum (Ogden, 2008; Agnew *et al*., 2009). Furthermore, as a result of marine resource scarcity, weak and/or poorly-enforced regulations and the drive to achieve greater profits or to sustain livelihoods, fishermen, fish suppliers and fish purveyors have frequently mislabelled their fish as higher-valued or more palatable-sounding species (Jacquet & Pauly, 2008). The mislabelling of fish species has recently been shown to be pervasive on a global scale (Marko *et al.*, 2004; Logan *et al.*, 2008; Wong & Hanner, 2008; Filonzi *et al.*, 2010; Miller & Mariani, 2010), the results of which have often held significant economic, conservation and even health impacts.

DNA sequencing methods have been employed in the field of fisheries biology for the identification of species and populations and their accuracy and reproducibility make such methods highly applicable for the monitoring and regulation of IUU fishing and fish species mislabelling (Unseld *et al*., 1995; Ogden, 2008). The success of such methods for species identification purposes, however, requires the extraction of suitable quantities of pure DNA from the tissue of interest, as well as the development of genetic databases containing reference DNA sequences from a wide range of fish species with which unknown sequences can be compared (Yue & Orban, 2001; Ward *et al*., 2009). Modern technology has greatly enhanced the ability to generate DNA sequence data, but global legislative bodies have been slow in adopting DNA-based techniques for fisheries monitoring and for ensuring regulatory compliance.

Although South Africa is among the most important fishing nations in Africa and in the world (INFOSA, 2007), the utilisation of DNA-based methods to authenticate fish species has not been extensively explored to date in this country. In addition, there is a lack or complete absence of reference sequence data in public genetic databases such as GenBank for many domestically available fish species. Until DNA-based methods are validated and comprehensive reference sequence databases are created, the accurate and unambiguous species-level identification of fishery products in South Africa using DNA sequencing methods will be precluded. Such a shortcoming will severely hamper the ability to optimally manage fisheries in this region and will curtail efforts to investigate and penalise IUU fishing activities and market fraud.

The overall aim of this study was to establish, compare and validate DNA-based methods for the identification of fish species commercially available in South Africa. Fulfillment of this aim entailed the meeting of several objectives, the first of which was to acquire a better understanding of the current trade dynamics of the South African fisheries market. In particular, accomplishment of this objective involved the performance of surveys in restaurants and retail outlets to determine the fish species most commonly marketed in this country, the state in which these species are most frequently sold and the quality of information presented on fish at the point of sale to enable consumers to make informed and sustainable seafood choices. Secondly, five DNA extraction methods were evaluated in order to identify the most appropriate method for the extraction of high yields of pure DNA from the muscle tissue of commonly marketed fish species in South Africa. The third objective was to compare different mitochondrial DNA markers (16S ribosomal RNA (rRNA), 12S rRNA and cytochrome *c* oxidase subunit I (COI) genes, as well as the control region in some cases) in terms of their ability to permit unambiguous species-level identification of 53 commercial fish species in South Africa, and to deposit the reference DNA sequences in international open-access databases such as GenBank and the Barcode of Life Database (BOLD). The final objective was to utilise the established DNA sequence database and the most suitable molecular methods to evaluate the extent of fish mislabelling and species substitution manifesting on the South African market.

References

Agnew, D., Pearce, J., Pramod, G., Peatman, T., Watson, R., Beddington, J. & Pitcher, T. (2009). Estimating the worldwide extent of illegal fishing. *PLoS One*, **4**, e4570.

Anyanova, E. (2008). Rescuing the inexhaustible (the issue of fisheries subsidies in the international trade policy). *Journal of International Commercial Law and Technology*, **3**, 147-156.

Clark, C. (2006). *The Worldwide Crisis in Fisheries: Economic Models and Human Behavior*. Pp. 1-29, 145-201. Cambridge, UK: Cambridge University Press.

Davies, R.W.D., Cripps, S.J., Nickson, A. & Porter, G. (2009). Defining and estimating global marine fisheries bycatch. *Marine Policy*, **33**, 661-672.

Delgado, C.L., Wada, N., Rosegrant, M.W., Meijer, S. & Ahmed, M. (2003). *The Future of Fish: Issues and Trends to 2020*. Pp. 1-5. Washington DC, USA: International Food Policy Research Institute and World Fish Center.

FAO (Food and Agriculture Organization of the United Nations) (2009). *The State of World Fisheries and Aquaculture 2008*. Pp. 1-175. Rome, Italy: FAO.

Filonzi, L., Chiesa, S., Vaghi, M. & Nonnis Marzano, F. (2010). Molecular barcoding reveals mislabelling of commercial fish products in Italy. *Food Research International*, **43**, 1383-1388.

INFOSA (2007). Fisheries in the SADC region: selected country profiles. [Internet document]. URL www.infosa.org.na/dloads/restrict/country%20profile/Fisheries-.pdf. 29 June 2010.

Jacquet, J.L. & Pauly, D. (2007). The rise of seafood awareness campaigns in an era of collapsing fisheries. *Marine Policy*, **31**, 308-313.

Jacquet, J.L. & Pauly, D. (2008). Trade secrets: renaming and mislabelling of seafood. *Marine Policy*, **32**, 309-318.

Jennings, S., Kaiser, M.J. & Reynolds, J.D. (2001). *Marine Fisheries Ecology*. P 6. Oxford, UK: Blackwell Science.

Logan, C.A., Alter, S.E., Haupt, A.J., Tomalty, K. & Palumbi, S.R. (2008). An impediment to consumer choice: overfished species are sold as Pacific red snapper. *Biological Conservation*, **141**, 1591-1599.

Marko, P.B., Lee, S.C., Rice, A.M., Gramling, J.M., Fitzhenry, T.M., McAalister, J.S., Harper, G.R. & Moran, A.L. (2004). Mislabelling of a depleted reef fish. *Nature*, **430**, 309-310.

Miller, D.D. & Mariani, S. (2010). Smoke, mirrors, and mislabeled cod: poor transparency in the European seafood industry. *Frontiers in Ecology and the Environment*, **8**, 517-521.

Ogden, R. (2008). Fisheries forensics: the use of DNA tools for improving compliance, traceability and enforcement in the fishing industry. *Fish & Fisheries*, **9**, 462-472.

Porter, G. (1998). *Estimating Overcapacity in the Global Fishing Fleets*. P 20. Washington DC, USA: WWF.

Sumaila, U.R., Khana, A., Watson, R., Munro, G., Zeller, D., Baron, N. & Pauly, D. (2007). The World Trade Organization and global fisheries sustainability. *Fisheries Research*, **88**, 1-4.

Unseld, M., Beyermann, B., Brandt, P. & Hiesel, R. (1995). Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. *PCR Methods and Applications*, **4**, 241-243.

Ward, R.D., Hanner, R. & Herbert, P.D.N. (2009). The campaign to DNA barcode all fishes, FISH-BOL. *Journal of Fish Biology*, **74**, 329-356.

Wong, H.K. & Hanner, R.H. (2008). DNA barcoding detects market substitution in North American seafood. *Food Research International*, **41**, 828-837.

Yue, G.H. & Orban, L. (2001). Rapid isolation of DNA from fresh and preserved fish scales for polymerase chain reaction. *Marine Biotechnology*, **3**, 199-204.

CHAPTER 2

LITERATURE REVIEW

A. Global trends in fish consumption, production and trade

Fish as a food source

The earliest interaction between man and the marine environment was as a result of the human appetite. More than 164 000 years ago, modern humans (*Homo sapiens*) are believed to have started consuming seafood on the coast of what is now South Africa, as evidenced by the findings of shell middens containing the remains of giant periwinkles, mussels and whelks (Marean *et al*., 2007). Since this time, the human race has increasingly looked to the ocean for fish as a source of food. Fish is highly nutritious, providing not only high-value protein, but also a wide range of essential micronutrients, minerals and fatty acids (Southgate, 2000). In many regions of the world, fish contributes substantially to food security. In developing countries, where animal protein intake is low, poor communities are highly reliant on fish to satisfy basic dietary requirements (McMichael & Butler, 2005). Fish provides more than 20% of the total protein intake for more than 2.6 billion people, equivalent to approximately 41% of the world population (Brunner *et al*., 2009). Around 22% of the protein intake in sub-Saharan Africa is supplied by fish (Béné & Heck, 2005). In economically developed countries, where incomes are typically high and basic dietary requirements are fulfilled, much attention is being given to the health benefits related to the consumption of fish (FAO, 2009a). Oily fish such as mackerel, salmon, anchovy, pilchard and herring are excellent sources of long-chain omega-3 fatty acids, including α-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Brunner *et al*., 2009). The specific roles of omega-3 fatty acids in preventing cardiovascular disease and cancer have been the subjects of active research and the basis for advice to increase the consumption of fish as part of a healthy diet (Brunner, 2006; Hooper *et al*., 2006; MacLean *et al*., 2006).

Fish consumption

The apparent world per capita fish consumption has nearly doubled in the last four decades from an average of 9.0 kg in the 1960s to an estimated 16.7 kg in 2006 (FAO, 2009a). Since 1961, total fish consumption has increased at a rate of 3.6% per year, outpacing the world population growth of 1.8% per year (WHO, 2003). The driving force behind the enormous surge in fish consumption appears to be due to a combination of population growth, rising incomes, urbanisation and dietary and lifestyle diversifications (Brunner *et al*., 2009; FAO, 2009a). However, as fish consumption has escalated over time, so too have concerns relating to the poor management and over-utilisation of the world's fish resources. As demand increasingly outstrips supply, fish has become more expensive relative to other marketed food items, negatively affecting food security and making fish protein unaffordable to some low-income families (Williams, 1996; Delgado *et al*., 2003).

Fish production and trade

In order to meet the world's burgeoning demand for fish, production of fishery products has soared (Delgado *et al*., 2003). In comparison to the 22 million metric tons of marine fish caught in 1948 (Rehbein & Oehlenschläger, 2009), the total global fishery capture in 2006 amounted to 143.6 million metric tons (FAO, 2009a). Approximately two thirds of the 2006 supply was derived from capture fisheries in marine and inland waters, while the remaining one third was from aquaculture (WHO, 2003; FAO 2009a). The rise in fish production, as with consumption, originates almost entirely from developing countries, which now generate nearly three times the volume of fish compared to developed countries (Delgado *et al*., 2003). Both capture fisheries and aquaculture, directly or indirectly, fulfil an indispensable role in the livelihoods of millions of people worldwide. In 2006, up to 43.5 million individuals were engaged in primary fish production and this number was as high as 170 million when those involved in secondary industries (processing, marketing and service) were included (FAO, 2009a).

In recent years, relaxing trade barriers and increasing globalisation of markets have led to fish becoming the most traded food commodity in the world (Béné, 2008). More than 37% of the global fish output (live weight equivalent) is currently traded across international borders (Delgado *et al*., 2003). Since 1996, the export of fish for human consumption has reportedly increased by 57% (FAO, 2009a). The European Union (EU), Japan and the United States (US) are importers of more than half of the world's fish supply by weight, equating to 77% by value (Valdimarsson, 2007). In 2006, an estimated 77% of the global fish supply was utilised for human consumption, while most of the remainder was reduced to fish meal and fish oil for livestock and carnivorous fish (aquaculture) feeds. Of the fish destined for human consumption, more than 48% was marketed in the preferred and highly priced fresh product form in

2006. The rest of the supply underwent some form of processing, 50% of which was frozen, 29% was marketed prepared or preserved and 21% was cured (FAO, 2009a).

B. The fishing industry in South Africa

Structure and characteristics of the industry

South Africa has a national fishing zone of approximately 688 926 km. The coastline stretches an estimated 3 000 km from Mozambique in the east, fed by the warm Agulhas current, to the shores of Namibia in the west, fed by the cold Benguela current. These waters offer a favourable habitat for more than 2 200 fish species, about 13% of which are endemic to South Africa. Industrial fishing is mainly concentrated on the highly productive west coast of South Africa, while recreational and subsistence fishing is more prevalent on the east coast, which has more species diversity but less biomass (Van der Elst, 1997; Branch & Clark, 2006).

The total South African fish capture in 2007 was estimated at 670 000 metric tons (live weight), the value of which was calculated at R4.5 billion per annum at this time (FAO, 2009b). Fisheries, however, remain a relatively small sector within the national economy, contributing approximately 1% of the total gross domestic profit (FAO, 2010). South African consumers reportedly spend around 4% of their total food budget on fish (Yakob *et al*., 2006). The per capita consumption of fishery products in South Africa is estimated at 8.6 kg per year (FAO, 2007). The domestic demand for fish is, however, not entirely satisfied by the local supply. On average between 2003 and 2005, South Africa imported more than 70 000 metric tons (live weight) of fish per annum in order to meet demands for species not fulfilled by the local catch or for exotic species not found in South African waters (FAO, 2008). Most fish imports were derived from America and the Asia Pacific (Yakob *et al*., 2006). Nonetheless, the quantity and value of imports into South Africa is far outweighed by that of exports of the local catch to international markets. Between 2003 and 2005, the total fish export from South Africa averaged 173 359 metric tons (live weight) per annum, equating to more than 37% of the total production (FAO, 2008). The main destination of these exports was Spain (33%) and Italy (17%) (Yakob *et al*., 2006).

Relative efforts in the fishing industry

Industrial fishing efforts in South Africa began in the late 1890s and escalated rapidly thereafter (FAO, 2010). Demersal trawl fisheries, dominated by deep-sea efforts for

Cape hakes (*Merluccius paradoxus* and *Merluccius capensis*), are the most important in terms of value, generating 47% of the total revenue of South African fisheries (DEAT, 2010). The demersal inshore trawl fishery, while contributing only 6% to the national hake catch, lands almost the entire valuable sole (*Austroglossus* spp.) catch. Retained bycatch of trawling includes kingklip (*Genypterus capensis*), Cape monk (*Lophius vomerinus*), mackerel (*Scomber* spp.), Cape dory (*Zeus capensis*) and gurnard (*Chelidonichthys capensis*) (SASSI, 2010). Pelagic purse-seine fisheries in South Africa, targeting predominantly pilchard (*Sardinops sagax*) and anchovy (*Engraulis* spp.), are second in term of value (20% of total revenue), but are the largest in terms of the volume landed (DEAT, 2010; FAO, 2010). Line fishing in South Africa contributes approximately 11% in value terms and mainly targets hake (*Merluccius* spp.), tuna (*Thunnus* spp.), snoek (*Thyrsites atun*), kob (kabeljou) (*Argyrosomus* spp.), yellowtail (*Seriola lalandi*) and geelbek (Cape salmon) (*Atractoscion aequidens*) (SASSI, 2010). At present, inland fisheries are not of commercial significance in South Africa and the commercial supply of freshwater fish is generated almost entirely by a limited number of aquaculture developments (FAO, 2010).

C. The state of world fisheries

Dwindling marine fish stocks

Unfortunately, the escalation of large-scale fishing activities around the world has caused widespread damage to marine ecosystems and has led to the collapse of innumerable fish populations (Myers & Worm, 2003; Hutchings & Reynolds, 2004; Pauly *et al*., 2005; Worm *et al*., 2006). Such a situation has arisen as a result of the open access and subsidy policies that have governed fisheries for many years and have created a ‘race for fish’ mentality within the industry (Pauly *et al*., 1998; Roheim & Sutinen, 2006). The exploitation of marine fish stocks increased rapidly during the 1970s and 1980s, spurred on by the increased investments in the sector and the expansion of fishing fleets, which nearly doubled in this time period (Delgado *et al*., 2003). As overfishing progressively removed larger predatory fish species from the ocean, efforts began to shift to smaller, low-trophic fish, leading to alterations in food web dynamics (Pauly *et al*., 1998; Pitcher & Pauly, 1998; Myers & Worm, 2003). By the late 1980s, many wild stocks had been fished at or beyond sustainable levels. In spite of the increased investment and fishing capacity, fish production has stagnated ever since (Delgado *et al*., 2003).

Illegal, unreported and unregulated (IUU) fishing has been identified as a major factor contributing to overfishing and the depletion of marine fish stocks (Worm *et al*., 2006). A prominent example of this has been the case of the Patagonian toothfish (*Dissostichus elegenoides*), which is now endangered as a result of illegal fishing and trading on the black market (NET, 2004, Knecht, 2006). In addition, IUU fishing undermines fishery management initiatives and results in the inaccurate estimation of prevailing fish stocks (Pitcher *et al*., 2002; Gallic & Cox, 2006). Ecological deterioration has not only been a result of excessive fishing, but also stems from the destructive methods used by many fishing sectors (Rubec, 1988; Lewison *et al*., 2004). Trawling and longline fisheries generate enormous amounts of bycatch (fish caught unintentionally by fishing gear), estimated annually at over 20 million tons and exceeding a quarter of the total fish catch (Delgado *et al*., 2003). Recent assessments suggest that over 75% of global fish stocks are now either fully exploited, overexploited or depleted (FAO, 2009a). It has further been reported that up to 90% of the ocean's large predatory fish, such as tuna and swordfish, are depleted (Myers & Worm, 2003). Based on the current trends, some marine ecologists have predicted the complete global collapse of all commercially important fish stocks by 2048 (Worm *et al*., 2006). Nonetheless, these predictions have been refuted by other scientific circles, who believe that threatened stocks may be successfully rebuilt through sound governance and incentives for fishing fleets (Hilborn *et al*., 2007; Branch, 2008).

Prospects for aquaculture

With pressure on wild fish stocks mounting, many countries have turned to aquaculture – the cultivation or 'farming' of aquatic organisms – as an alternative means of producing marine and freshwater fish for human consumption (Martinez *et al*., 2005; FAO, 2007). Aquaculture has expanded more rapidly than any other animal protein-producing sector in the world (FAO, 2009a). In 2006, aquaculture contributed 47% of the world's total fish production and 20% of the total marine fish production (Brunner *et al*., 2009). While aquaculture was originally anticipated to be a sustainable manner of easing pressure on marine fish stocks, the practice has come under immense scrutiny due to its potentially negative impacts on the environment (Tidwell & Allan, 2001; Delgado *et al*., 2003). One widely publicised issue has been the destruction of thousands of hectares of mangrove forests to make way for the development of aquaculture ponds, resulting in extensive habitat losses in many coastal regions (De Graaf & Xuan, 1998; Alongi, 2002). In addition, the effluent generated by intensive aquaculture production (containing biological waste, hormones and pesticides) is often released directly into adjoining waterways, polluting the surrounding environment (De Walt *et al*., 1996). Farmed fish that escape from pens into the wild can compete with wild stocks for resources, act as predators of native species, or even interbreed and modify the genetic pools of wild populations (Delgado *et al*., 2003). Much criticism has also been focused on the utilisation of fishmeal in aquaculture diets, which is derived predominantly from oily marine pelagic fish (Tidwell & Allan, 2001; Brunner *et al*., 2009). These small fish are vital sources of food for wild marine predators and their exploitation for aquaculture can disrupt marine food web systems (Delgado *et al*., 2003). Naylor *et al*. (1998) reported that some carnivorous fish, such as salmon, require up to 3 kg of wild fish in their diets to produce 1 kg of farmed fish. Thus, unless aquaculture can be decoupled from marine fishing, it is unlikely to solve the dilemma of maintaining a sustainable fish supply (Naylor *et al*., 2000; Brunner *et al*., 2009).

D. The sustainable seafood movement

The emergence of sustainable seafood initiatives

At a time when conventional governmental command-and-control policies are appearing to be ineffective in reversing global fishery collapses, a growing number of private sector, non-governmental organisations (NGOs), mostly environmental groups, are bypassing the state by launching sustainable seafood campaigns to promote more effective fisheries management (Hannesson, 1996; Wessells *et al*., 1999; Illes, 2004). The primary aim of such movements is to educate and create market awareness about sustainable seafood choices using concise, scientifically-based information. The use of logos or 'eco-labels' has become a popular means of conveying to consumers that a product complies with certain environmental standards. From a policy standpoint, the aim of eco-labelling is to modify consumer purchasing behaviour and to increase the demand for sustainable fish products. From a business standpoint, eco-labelling motivates fish producers to use more sustainable resources and 'environmentally-friendly' production methods, with the expectation of acquiring a greater market share and higher profits (Phillips *et al*., 2003; Roheim, 2003; Kaiser & Edwards-Jones, 2006).

International sustainable seafood initiatives

Perhaps the most well-established sustainable seafood initiative is the Marine Stewardship Council (MSC), which operates internationally as an independent, nonprofit organisation. The MSC was originally formed in 1997 via collaboration between the World Wildlife Fund (WWF), a major international environmental NGO, and Unilever, one of the world's largest fish retailers. The main objective of the MSC is to promote and reward environmentally responsible fisheries management by means of market-based incentives, including certification and eco-labelling (Constance & Bonanno, 2000; Phillips *et al*., 2003). Fishery products are not permitted to bear the blue MSC eco-label until they have been certified according to two sets of criteria, both of which are audited by independent third party certification bodies appointed by the MSC (Constance & Bonanno, 2000; Cummins, 2004). The first tier of certification requires that a fishery is deemed to be sustainable according to the MSC's 'Principles and Criteria for Sustainable Fisheries'. The second tier involves chain-of-custody certification, which requires that certified products are kept separated from non-certified ones and that these can be traced back through the entire supply chain, from the point of sale to the fishery of origin (Roheim & Sutinen, 2006; Ponte, 2008; Gulbrandsen, 2009). The MSC website (www.msc.org) indicates that 106 fisheries worldwide have been certified and a further 145 fisheries are currently undergoing assessment at the present time (April 2011). The South African hake trawl fishery was the first fishery in Africa to obtain MSC certification in 2004 and was re-certified as sustainable in 2010.

Apart from the MSC certification initiatives, a number of alternative market- and consumer-based sustainable seafood campaigns have emerged globally in the last 12 years. Single-species campaigns launched in the US have included the 'Give Swordfish a Break' campaign, which urged restaurant chefs and consumers to stop purchasing the overfished swordfish (SeaWeb, 2002) and the 'Take a Pass on Chilean Sea Bass' campaign, aimed at protecting the slow-growing and heavily-exploited Patagonian toothfish (Roheim & Sutinen, 2006). Other consumer-directed campaigns have shifted away from these 'boycott' strategies, advocating that the public consume fish in a sustainable manner, rather than completely refrain from its consumption (Iles, 2004). Organisations such as the Monterey Bay Seafood Watch, Environmental Defense, the Audubon Society and SeaWeb's globally-directed Seafood Choice Alliance have popularised the use of sustainable seafood lists and colour-coding guides to differentiate seafood products according to sustainability criteria (e.g. 'best choice' or 'avoid') (Gulbrandsen, 2009). The details of these programmes are typically disseminated to consumers by means of food product labels, web-accessible databases, wallet cards and mobile phone applications (Iles, 2004; Kinkade & Verclas, 2008).

Southern African Sustainable Seafood Initiative (SASSI)

Launched in late 2004 under the banner of the WWF, the Southern African Sustainable Seafood Initiative (SASSI) operates according to similar principles as other international seafood consumer awareness campaigns (Jacquet & Pauly, 2007; 2008a; Von der Heyden *et al*., 2010). At its core, SASSI aims to create awareness about marine conservation issues among participants of the fishing industry and consumers and to promote industry compliance with the prevailing South African fisheries regulations (Marine Living Resources Act, Act No. 18 of 1998) (SASSI, 2010). The SASSI consumer seafood list (Table 1) guides consumers to make the most sustainable choices from commonly encountered fish in South Africa using a traffic-light approach to rank species, ranging from green ('best choice') to red ('worst choice'). The rankings are based on abundance, conservation and legal status criteria. SASSI has made this sustainable seafood list available to the public via its web-accessible database (www.wwfsassi.co.za), pocket cards and, since 2007, as a mobile phone text message service known as 'FishMS' (Von der Heyden *et al*., 2010).

Limitations and criticisms of sustainable seafood initiatives

In spite of the good intentions of the sustainable seafood movement, its global growth has not occurred without criticism. While single-species campaigns have increased consumer awareness on selected dwindling fish populations, is has been pointed out that focusing on a single facet of environmental protection will not successfully address the major environmental problems existing in the fisheries sector (Jacquet & Pauly, 2007). Consumer awareness campaigns and eco-labelling initiatives have also been criticised for tending to target large-scale fisheries and for being somewhat limited in their geographical scope (Jacquet & Pauly, 2008b; Jacquet *et al*., 2009). The high prevalence of international fish trade limits the relevance of seafood lists that only include local species, and necessitates regular updating of lists as new species become available on seafood markets (Von der Heyden *et al*., 2010). In addition, since the effects of such campaigns are highly reliant on consumer education, access to information and environmental involvement, controversy has arisen on the impact of such movements on overall consumer behaviour (Teis *et al*., 1999; Rex & Baumann, 2007). For example, while more than 65% of the global fish supply is consumed in Asia, most Asian consumers do not discriminate between fishery products based on their environmental impacts and do not readily respond to consumer awareness campaigns (Jacquet & Pauly, 2008a).

Table 1 SASSI consumer list of commonly encountered fish species in South Africa rated according to sustainability criteria (as of 01 August 2010) (SASSI, 2010)

Green list – best choice[1]		Orange list – caution[2]		Red list – no sale[3]	
Common name	**Scientific name**	**Common name**	**Scientific name**	**Common name**	**Scientific name**
Anchovy	*Engraulis encrasicolus*	Bigeye tuna	*Thunnus obsesus*	Baardman	*Umbrina* spp.
Angelfish	*Brama brama*	Bluefin tuna	*Thunnus maccoyii*	Banded galjoen	*Dichistius multifasciatus*
Blue hottentot	*Pachymetopon aeneum*	Carpenter seabream	*Argyrozona argyrozona*	Blacktail	*Diplodus sargus capensis*
Bluefish / bluenose	*Hyperoglyphe antarctica*	Dageraad seabream	*Chrysoblephus cristiceps*	Brindle bass	*Epinephelus lanceolatus*
Blueskin seabream	*Polysteganus coeruleopunctatus*	Dusky kob	*Argyrosomus japonicus*	Bronze bream	*Pachymetopon grande*
Bonito	*Sarda* spp.	East coast sole	*Austroglossus pectoralis*	Cape knifejaw	*Oplegnathus conwayi*
Butterfish	*Ruvettus pretiosus / Lepidocybium flavobrunneum*	Elf / shad	*Pomatomus saltatrix*	Cape stumpnose	*Rhabdosargus holubi*
Chub mackerel	*Scomber japonicus*	Englishman seabream	*Chrysoblephus anglicus*	Galjoen	*Dichistius capensis*
Dorado	*Coryphaena hippurus*	Geelbek / Cape salmon	*Atractoscion aequidens*	Garrick	*Lichia amia*
Gurnard	*Chelidonichthys* spp.	King mackerel	*Scomberomorus commerson*	Janbruin	*Gymnocrotaphus curvidens*
Hake	*Merluccius paradoxus / M. capensis*	King soldier seabream	*Argyrops spinifer*	Kingfish	*Carangoides* /*Caranx* spp.
Horse mackerel	*Trachurus capensis*	Kingklip	*Genypterus capensis*	Largespot pompano	*Trachinotus botla*
Hottentot	*Pachymetopon blochii*	Marlin	*Makaira / Tetrapturus* spp.	Natal knifejaw	*Oplegnathus robinsoni*
Jacopever	*Helicolenus dactylopterus*	Poenskop	*Cymatoceps nasutus*	Natal stumpnose	*Rhabdosargus sarba*
John Dory	*Zeus* spp.	Red stumpnose	*Chrysoblephus gibbiceps*	Natal wrasse	*Anchichoerops natalensis*
Longfin tuna	*Thunnus alalunga*	Red/copper steenbras	*Petrus rupestris*	Potato bass	*Epinephelus tukula*
Monkfish	*Lophius vomerinus*	Rockcods	*Epinephelus* spp.	River bream	*Acanthopagrus berda* /*A. vagus*
Mullet / harders	*Liza spp. / Mugil* spp.	Roman seabream	*Chrysoblephus laticeps*	River snapper	*Lutjanus argentimaculatus*
Panga seabream	*Pterogymnus laniarus*	Sailfish	*Istiophorus* spp.	Sawfish	*Pristis* spp.
Queen mackerel	*Scomberomorus plurilineatus*	Scotsman seabream	*Polysteganus praeorbitalus*	Seventy-four	*Polysteganus undulosus*
Ribbon snoek	*Lepidopus caudatus*	Silver kob	*Argyrosomus inodorus*	Southern pompano	*Trachinotus africanus*
Sand soldier	*Pagellus bellottii natalensis*	Slinger seabream	*Chrysoblephus puniceus*	Spotted grunter	*Pomadasys commersonni*
Santer seabream	*Cheimerius nufar*	Snappers and jobfish	Family *Lutjanidae*	Springer	*Elops machnata*
Sardine/pilchard	*Sardinops sagax*	Squaretail kob	*Argyrosomus thorpei*	Stonebream	*Neoscorpis lithophilus*
Skipjack tuna	*Katsuwonus pelamis*	Swordfish	*Xiphias gladius*	West coast steenbras	*Lithognathus aureti*
Snoek	*Thyrsites atun*	West coast sole	*Austroglossus microlepis*	White musselcracker	*Sparodon durbanensis*
Steentjie seabream	*Spondyliosoma emarginatum*			White steenbras	*Lithognathus lithognathus*
White stumpnose	*Rhabdosargus globiceps*			Zebra	*Diplodus curvinus hottentotus*
Yellowfin tuna	*Thunnus albacares*				
Yellowtail	*Seriola lalandi*				

[1] Green-listed species are those from relatively healthy, well managed fish populations that are likely to handle current fishing pressures; recommended as the most sustainable choices available.
[2] Orange-listed species are those that are currently overexploited, vulnerable to overfishing or are caught using environmentally problematic methods; recommended to be considered with caution.
[3] Red-listed species are those that are illegal to buy or sell in South Africa as these are recreational or specially protected species.

Unlike the certification schemes of the MSC, the colour-coding and/or seafood-ranking guides used in consumer awareness campaigns generally do not entail environmental standard setting and third-party audits of the fish supply chain (Iles, 2004, Jacquet & Pauly, 2007). The problem arising in this context is that sustainable seafood recommendations are only as effective as the information or product labelling which is provided to the consumer at the point of sale (Von der Heyden *et al*., 2010). The widespread renaming and mislabelling of fish species on the market can lead to poor choices being inadvertently made by concerned, but uninformed, consumers (Jacquet & Pauly, 2007; Logan *et al*., 2008). It has been suggested that such initiatives need to focus more attention on improving labelling standards and compliance through authentication testing to ensure their effectiveness (Jacquet, 2009).

E. Market substitution of fish species: incidence and consequences

Factors hampering accurate fish species authentication

The current nature of the global fisheries market presents a number of challenges for the accurate identification of fish products at the species level (Martinez *et al*., 2005). At present, the variety of fish consumed by humans is vast, comprising up to 5 000 different species (Froese & Pauly, 2010). As many of the preferred fish for human consumption become increasingly rare, fisheries are likely to seek alternative, underutilised species to exploit, which will inevitably add to this total (Delgado *et al*., 2003). Another major problem with species authentication is the increasing demand for processed fish products. Fish species can generally be identified by knowledgeable individuals when the specimens are in their whole state (Gil, 2007). However, once filleting removes the distinctive morphological characteristics required for identification (head, skin, fins and bones), identification by visual inspection becomes difficult (Martinez *et al*., 2005). Additional value-added processing such as mincing, coating and frying complicate the matter even further. The flesh of many fish species differs only subtly in flavour and texture and it is therefore often difficult for consumers to identify the species when fish has been processed or prepared for consumption (Buck, 2009). The international trading of fish commodities adds to the authentication burden. As alternatives for dwindling local marine fish stocks, many foreign or farmed species may be introduced into markets which would otherwise be unfamiliar with them (FAO, 2000). In essence, the sum of these factors set up a fisheries market that is conducive to the deliberate or unintentional substitution of low-valued fish for higher-valued ones.

Renaming and mislabelling

As the global market and international trade opportunities for seafood have grown, so to have the associated problems of renaming and mislabelling of species. Financial incentives have been the main motivators for many fish being provided with entirely new names, often which are similar to those of already popularised fish species (Jacquet & Pauly, 2008a). Many fish, such as the stumpknocker (*Lepomis punctatus*) and hogsucker (*Hypentelium* spp.), were originally afforded names which would understandably not be well accepted by modern-day consumers. Consequently, the US National Marine Fisheries Service (NMFS) expended $8.5 million between 1973 and 1981 to investigate which underutilised species with a poor image should be renamed (Miller, 1981). Originally called 'slimehead' in 1957, this fish (*Hoplostethus atlanticus*) was prudently renamed 'orange roughy' as the market for seafood developed (Pauly *et al*., 2003). With permission from trade authorities, the British grocer Marks & Spencer renamed 'witch' (*Glyptocephalus cynoglossus*) as 'Torbay sole' to broaden its appeal (Dobson & Mesure, 2007). Under the US Food and Drug administration (FDA) regulations, 13 species of Pacific rockfish (*Sebastes* spp.) are permitted to be marketed as 'Pacific red snapper' when they are sold through interstate commerce in California, Oregon and Washington (Randolph & Snyder, 1993). Pacific rockfishes are, however, an ecologically diverse genus comprising around 60 different species (Love *et al*., 2002). In one study, Logan *et al*. (2008) found that 56% of the rockfish species marketed in the US were listed as overfished by the NMFS. Thus, the consent by state and federal agencies to allow rockfishes to be marketed under a single vernacular name compromises the consumer's ability to differentiate the species based on their conservations statuses. Ambiguities have also arisen from the use of vernacular names such as 'whitefish', which may include Alaskan pollock, halibut, cod or sole. Additionally, products generically labelled as 'salmon' may include various salmon species, many of which are endangered (Hold *et al*., 2001; IUCN, 2006).

While many new names have appeared on the market, not all of these have been officially accepted for use. For example, although the Patagonian toothfish was renamed 'Chilean sea bass' by a fish merchant in the 1970s, the FDA refused to authorise this change based on the fact that the Patagonian toothfish does not fall into the sea bass family (Knecht, 2006). Attempts in the 1990s to rename tilapia as 'St. Peter's fish' (intended to relay the fact that the fish is found on the Sea of Galilee in Israel) were also refuted by the FDA (Foulke, 1993). Other renaming strategies have been used loosely on the market, often without any attempts at official recognition.

Malabar blood snapper (*Lutjanus malabaricus*) and South African Cape hakes (*M. capensis* and *M. paradoxus*) are now flouted on international markets as 'Scarlet snapper' (Walsh, 2001). Basa (*Pangasius bocourti*) from Vietnam has also been sold as 'white roughy', 'Pacific dory' and 'Cajun delight' (Laws, 2001; Nohlgren, 2006).

Although the renaming of fish may hinder authentication, the problems associated with these practices are far eclipsed by the magnitude of those caused by the mislabelling of fish as different species (Jacquet & Pauly, 2008a). While the substitution of species may be deliberate for some fish due to their differing values, it may also occur accidentally when species identities are easily mistaken (Buck, 2009). Nonetheless, regardless of the circumstances surrounding the mislabelling of seafood products, the potential consequences (economical, environmental and health impacts) are often equally damaging. Problems can arise from misrepresentation of fish species at the restaurant or retail level, substitution by suppliers or wholesalers, or the misidentification of products at any stage in the harvesting and production systems. A great number of instances of fish misnaming or mislabelling have been published in the literature (Table 2), most of which have involved the substitution of less expensive, low quality species for higher priced, higher quality, or more palatable sounding species (Logan *et al*., 2008). Routine examinations carried out by the NMFS National Seafood Inspection Laboratory over a nine-year period (fiscal years 1988 - 1997) showed that 37% of fish products from randomly selected vendors in the US were mislabelled (Tennyson *et al*., 1997). More recently, DNA analyses conducted on fish products obtained from restaurants and markets in New York showed that 14 of the 56 (25%) fish samples studied were incorrectly labelled as higher-priced species (Buck, 2009).

Consequences of fish mislabelling

Financial losses - consumers and government

As a result of resource scarcity, the price for certain fish species is high. In order to circumvent hefty expenditures, it has been revealed that distributors, retailers and restaurants may purchase lower-valued species, substitute them with their higher-valued counterparts, and reap the resulting profits (Jacquet & Pauly, 2008a). Over the last two decades, a wide variety of different fish species have masqueraded as 'the' red snapper (*Lutjanus campechanus*), a highly-prized eating fish found in the southern Atlantic and Gulf of Mexico (Table 2). When up to 77% of the fish sold as red snapper in the US were substituted with less valuable species (Marko *et al.,* 2004), it was the consumer that lost from the substitution, not the retailer. Similar problems have occurred with red emperors (*Lutjanus sebae*) in Australia, where around 40% of such products have been substituted with cheaper species (Table 2).

In 1989, the FDA intercepted a 20 400 kg shipment of Oreo dory (*Pseudocyttus maculatus*) labelled as orange roughy (*Hoplostethus atlanticus*) on route to Ohio from New Zealand, which would have probably sold for three times its value (Foulke, 1993). Consumers in Chicago would likely have been upset to know that, on about 70% of occasions, they paid up to four times higher prices for grouper (*Epinephelus* spp. and *Mycoteroperca* spp.) that was substituted with lower-valued species such as hake, catfish and tilapia. The latter species not only fall outside any genus of grouper, but also fall outside the wider family of sea basses to which grouper belong (Heemstra & Randall, 1993). In South Africa, it is reportedly not uncommon for unscrupulous vendors to trim the tails of kob (*Argyrosomus* spp.) so that they resemble the more expensive geelbek (*Atractoscion aequidens*). One DNA-based study revealed that up to 84% of fillets marketed as 'kob' in South Africa were, in fact, from other species, including mackerel, croakers and warehous (Von der Heyden *et al*., 2010).

Environmental impacts

The renaming or mislabelling of fish undermines environmental regulations and poses a serious threat to species that are exploited, specially protected or illegal to sell (Jacquet & Pauly, 2008a). The renaming of the Patagonian toothfish (*Dissostichus eleginoides*) as ''Chilean sea bass'' in the 1970s successfully enhanced its market appeal, however, increased demand soon led to the overfishing of the species, 80% of which was estimated to be illegal in 1999 (Lack & Sant, 2001; Knecht, 2006). Illegally harvested toothfish has entered the market labelled under the non-descript term 'frozen fish fillets' or even labelled as or mixed with other seafood products. Such deceptive practices led to the 2001 South African indictment of the Hout Bay Fishing Industries, who endeavoured to smuggle two tons of illegally harvested Patagonian toothfish into the US underneath a layer of crayfish (NET, 2004). In addition, Clover (2006) reported that once quotas for the now heavily exploited cod (*Gadus* spp.) have been exceeded, English fisherman mislabel the catch as 'ling' to get it through customs. Conversely, many other fish species have been fraudulently sold as 'cod' (Table 2), creating the perception to consumers that supply can keep up with demand. Thus, as was the case with cod, the collapse of fish stocks in other parts of the world will likely go unnoticed if they are perceived to be readily available on retail shelves (Jacquet & Pauly, 2008a).

Table 2 Documented cases of the fraudulent mislabeling of fish products aimed at deliberately deceiving consumers

Labeled as		Identified substitute		Country	Comments	Reference
Common name	**Scientific name[1]**	**Common name**	**Scientific name**			
Barramundi	*Lates calcarifer*	Nile perch	*Lates niloticus*	AUS	13% of 'barramundi' samples tested were mislabelled	FSANZ, 2003
Barramundi	*Lates calcarifer*	King threadfin	*Polydactylus macrochir*			
Cod	*Gadus morhua*	Alaska pollock	*Theragra chalcogramma*	US		Burros, 1992
Cod	*Gadus morhua*	Oilfish	*Ruvettus pretiosus*	HK	After ingesting mislabeled 'cod', consumers were hospitalised with severe diarrhea	Lam, 2007
Flounder	Pleuronectidae	South Pacific hake	*Merluccius gayi*	EC		Martinez-Ortiz, 2005
Flounder	Pleuronectidae	Shark	Selachians	EC		Bostock & Herdson, 1985
Grouper	*Epinephelus / Mycteroperca* spp.	Spangled emperor	*Oreochromis* spp.	US	Up to 70% of tested 'groupers' were mislabelled	Nohlgren & Tomalin, 2006; 2007; Reed, 2006
Grouper		Channel catfish	*Ictalurus punctatus*	US		
Grouper		Hake	*Merluccius* spp.	US		
Grouper		Alaska pollock	*Theragra chalcogramma*	US		
Dorado	*Coryphaena hippurus*	Yellowtail	*Seriola lalandi*	US, SA		FDA, 2009a; Von der Heyden *et al*., 2010
Kob / kabeljou	*Argyrosomus* spp.	Silver warehou	*Seriolella punctata*	SA	84% of the 'kob' fillets tested in South Africa were mislabelled	Von der Heyden *et al*., 2010
Kob / kabeljou	*Argyrosomus* spp.	Bigscale mackerel	*Gasterochisma melampus*			
Kob / kabeljou	*Argyrosomus* spp.	Blackspotted croaker	*Protonibea diacanthus*			
Monkfish	*Lophius* spp.	Pufferfish	*Tetrodon* spp.	US	Two Chicago customers fell ill from tetrodotoxin in pufferfish mislabelled as 'monkfish'	Cohen *et al*., 2009
Orange roughy	*Hoplostethus atlanticus*	Oreo dory	*Pseudocyttus maculatus*	US		FDA, 2009a
Orange roughy	*Hoplostethus atlanticus*	John dory	*Zeus faber*	US		
Patagonian toothfish	*Dissostichus eleginoides*	Antarctic toothfish	*Dissostichus mawsoni*	US		Knecht, 2006
Chum salmon	*Oncorhynchus keta*	Pink salmon	*Oncorhynchus gorbuscha*	US		FDA, 2009a
Red snapper	*Lutjanus campechanus*	Rockfish	*Sebastes* spp.	US	70–80% of 'red snappers' sold in the US were mislabeled	Hsieh *et al*., 1995; Tennyson *et al*., 1997; Marko *et al*., 2004; FDA, 2009a
Red snapper	*Lutjanus campechanus*	Tilapia	*Oreochromis* spp.	US		
Red snapper	*Lutjanus campechanus*	Dorado	*Coryphaena hippurus*	US		
Red snapper	*Lutjanus campechanus*	Channel catfish	*Ictalurus punctatus*	US		
Red emperor	*Lutjanus sebae*	Spangled emperor	*Lethrinus choerorhynchus*	AUS	41% of 'red emperors' tested were mislabelled	FSANZ, 2003
Red emperor	*Lutjanus sebae*	Red throat emperor	*Lethrinus miniatus*	AUS		
Swordfish	*Xiphias gladius*	Mako shark	*Isurus oxyrinchus*	US		FDA, 2009a
Tilapia	*Oreochromis* spp.	South Pacific hake	*Merluccius gayi*	EC		Martinez-Ortiz, 2005
'Wild' salmon	*Oncorhynchus* spp.	Farmed salmon	*Salmo salar*	US		Burros, 2005

[1]The scientific names of fish were verified using the information in FishBase (www.fishbase.org)
Abbreviations: AUS = Australia; EC = Ecuador; HK = Hong Kong; SA = South Africa; US = United States

Undermining of seafood awareness campaigns

Central to the success of all consumer choice campaigns is the requirement for the accurate labelling of seafood products in the marketplace (Logan *et al*., 2008). Renaming or mislabelling not only deceives active consumers into thinking they are making 'eco-aware' purchases, but also dejects from the campaigns intentions to promote sustainability (Jaquet & Pauly, 2007). Unfortunately, some deceitful, profit-driven exporters and/or domestic suppliers have capitalised on the lack of traceability that exists in many seafood supply chains to promote their products as eco-friendlier versions. For example, in 2003, a grocery store in Washington was caught fraudulently mislabelling 4 - 5% of their fish with the 'EcoFish' label - a certification sticker that signifies that a product was harvested in an environmentally responsible way (Denn, 2003). The eco-friendly reputation of tilapia was a factor contributing to its increased popularity in the US. However, this reputation was tarnished when the Whitefish Association of Ecuador began selling South Pacific hake labelled as tilapia (Martinez-Ortiz, 2005). In 2001, the 'Farmed and Dangerous' campaign was initiated by the Coastal Alliance for Aquaculture Reform to discourage the consumption of farmed salmon due to the adverse impacts of their production on the environment (Jaquet & Pauly, 2008a). Such campaigns, however, have little impact when farmed salmon is intentionally mislabelled on the market as 'wild' (Table 2).

Health impacts

It has long been recognised that the high indigestible wax ester content of escolar (*Lepidocybium flavobrunneum*) and oilfish (*Ruvettus pretiosus*) has purgative effects, frequently causing outbreaks of oily diarrhoea (keriorrhea) following consumption (Cox & Reid, 1932; Mori *et al*., 1966; Berman *et al*., 1981; Givney, 2002; Gregory, 2002; Feldman *et al*., 2005). However, due to the frequent occurrence of these two species as a bycatch of the swordfish and tuna fisheries (Shadbolt *et al*., 2002; Tserpes *et al*., 2006), both fish continue to be actively marketed in many countries. Bans on the sale and import of escolar and oilfish have been placed in three countries (Italy, Japan and South Korea), while only guidelines regarding their trade and consumption have been issued by the US, Australia, Canada and many European member states (Alexander *et al*., 2004). The fish labelling regulations of the European Communities (EC, 2003a) stipulates that *L. flavobrunneum* and *R. pretiosus* must be marketed as escolar and oilfish, respectively. No such regulations exist in South Africa and both species are frequently marketed as 'butterfish' in this country (Von der Heyden *et al*., 2010).

Escolar has also been sold under the name of 'white tuna' or 'snowfish' in China (Mok, 2007) and as 'rudderfish' in South Africa (Berman *et al*., 1981). In 2006, consumers in Hong Kong received a large shipment of oilfish steaks from Indonesia which were marketed as 'Atlantic cod'. This potent case of mislabelling resulted in an outbreak of more than 600 cases of keriorrhea (Chong, 2007; Lam, 2007) (Table 2).

In another case, two Chicago consumers were hospitalised after ingesting the tetrodotoxin found in poisonous pufferfish, which was mislabelled as harmless 'monkfish' (Cohen *et al*., 2009) (Table 2). The accurate labelling of fish species is also imperative in terms of contaminants, particularly mercury, which accumulate in the flesh of predatory fish, such as tuna (Jaquet & Pauly, 2008a). About 90 million cans of 'light tuna' sold annually in the US actually contain yellowfin tuna (*Thunnus albacares*) or albacore (*Thunnus alalunga*), which have a three times higher mercury content than the skipjack tuna (*Katsuwonus pelamis*) expected in these cans (Burger & Gochfeld, 2004; Roe & Hawthorne, 2005). The importance of country of origin labelling in relation to human health was emphasised when high pesticide residues were found in Chinese shellfish (Guo *et al*., 2007) and when salmon raised in Europe were shown to contain higher contaminant levels than those from North and South America (Hites *et al*., 2004).

The consumption of seafood products with parasitic (*Anisakis* spp.) contamination and elevated histamine levels have been implicated in the occurrence of immune-mediated allergic reactions and non-immune intolerance reactions, respectively (Saavedra-Delgado & Metcalfe, 1993; Montoro *et al*., 1997, Moreno-Ancillo *et al*., 1997). Worldwide, fish and shellfish are recognised as two distinct major food allergens, both with the potential to cause severe reactions from the consumption of even milligram-level amounts (Sackesen & Adalioglu, 2003; Sampson, 2004). In South Africa, allergic reactions have occurred following the consumption of hake, yellowtail, salmon and mackerel, as well as prawns, crayfish, abalone and black mussels (Zinn *et al*., 1997). The accurate labelling of seafood species is therefore imperative to allow allergic consumers to avoid products which may have an adverse effect on their health.

Legislation relating to the labelling of fishery products

The legislation of many countries, including South Africa, secures the right of consumers to be informed about the contents of food products through the packaging labels and stipulates that this labelling should not be misleading (DJC, 1985a; 1985b; FSANZ, 1991; EC, 2000a; FDA, 2005; DTI, 2009; DOH, 2010). The European Communities (EC) have perhaps taken the most vigilant approach in informing consumers on fish products. Regulations implemented by the EC specify that fishery and aquaculture products may not be offered for sale unless they are labelled with their designated name (commercial and Latin names), geographical origin, production method (wild or farmed) and whether they contain genetically modified material (EC, 2000b; 2001; 2003b). On a global scale, the United Nations Food and Agricultural Organisation (FAO) Codex Alimentarius (1985) requires that the country of origin of all food products be declared, with the exception that 'when a food undergoes processing in a second country which changes its nature, the country in which the processing is performed shall be considered to be the country of origin'. In terms of the naming of fish products, however, international trade has made uniformity problematic under these regulations. There have been differences in opinions among the more than 190 FAO member countries regarding which fish species can share common market names (Jacquet & Pauly, 2008a). For instance, the EU and Peru have disagreed on exactly which fish species constitutes a 'sardine' (EC, 2002).

In the US, imported seafood is controlled by the FDA, but the regulation of the labelling of seafood is the responsibility of the US Department of Agriculture (USDA). The USDA has developed country of origin labelling (COOL) regulations, which specify which products must be labelled, who is required to supply the labels and what information is required on these labels. The COOL regulations went into effect for fish and shellfish products on April 5, 2006, the final specifications of which were published on January 15, 2009 (USDA, 2009). Recognising the confusion that can arise with fish naming during international trade, the FDA has published a 'seafood list' which includes approved market names (and scientific names) for a wide range of local and imported finfish and shellfish commonly sold in the US (FDA, 2009b). The primary aim of this list is to promote uniformity and to establish order in the marketplace, while reducing confusion among consumers (Randolph & Snyder, 1993; Martinez *et al*., 2005).

In South Africa, the issuance and enforcement of regulations pertaining to the labelling of fishery products falls predominantly under the jurisdiction of the Department of Health (DoH) and the Department of Trade and Industry's (DTI's) regulatory division, known as the National Regulator for Compulsory Specifications (NRCS). Both the regulations relating to the labelling and advertising of packaged foodstuffs (DoH, 2010) and the NRCS regulations for frozen fish products (DTI, 2003) specify that the country of origin of foodstuffs must be declared on product labels. Although both of the aforementioned sets of regulations recognise that the naming and/or labelling of fishery products should not mislead the consumer, neither require the declaration of fish by designated or Latin names or the qualification of such products as wild caught or farmed. The reported high frequency of fish fraud on the South African market (Von der Heyden *et al*., 2010) may thus be correlated with the lack of South African legislative requirements to label fish by designated names (as in the EU) and the lack of guidelines to assist the process (e.g. seafood lists), the lack of traceability requirements and the lack of accurate analytical methods to authenticate the marketed fish products.

F. Methods for fish species authentication

Identification of fish at the species level relies on the examination of either external or internal characteristics that are inherent to particular species (Bossier, 1999; Gil, 2007). Sensory analysis has been utilised in some cases as an attempt toward fish speciation, however, the sensory profiles of closely related species are difficult to characterise, even by trained panels of individuals (Suvanich *et al.*, 2000). With the prospects of growing international trade and the increased use of processing methods which remove the distinguishing external features required for fish species identification, rapid and accurate analytical methods are required to distinguish fish species based on their unique internal characteristics (Céspedes *et al*., 1998; McDowell & Graves, 2002). Although a variety of methods are available for fish species authentication (Table 3), the most appropriate choice in each case depends on the nature of the product, including whether the fish is whole or processed, raw or heated, or comprising single or multiple species (Rehbein & Horstkotte, 2003; Sotelo *et al.*, 2003). Traditional methods for species identification are typically based on the analysis of proteins or DNA which are contained in the product (Martinez, 2003; Hubalkova *et al*., 2007). Both protein- and DNA-based methods can be divided into two groups: those that target one or a few loci or those that target many loci to produce a fingerprint-like pattern (Table 3).

Protein-based approaches to fish species identification

Most early research aimed at the identification of fish products focused on the use of proteomic techniques, particularly electrophoretic, chromatographic and immunological methods, to exploit the physicochemical differences in the protein structures of closely related species (Piñeiro *et al.*, 1999; Hubalkova *et al*., 2007). Electrophoresis involves the separation of water-soluble proteins according to their molecular weights under the influence of an electric field. Separation is accomplished within a gel matrix, normally agarose or polyacrylamide (Walker, 2003). Various forms of electrophoresis may be used depending on the type and nature of the fish product to be analysed (Table 3).

Protein isoelectric focusing (IEF), which separates proteins based on their isoelectric points (pI's), was the first analytical method used for fish species identification (Lundstrom, 1980) and was later accepted as an official method for this purpose (AOAC, 1995). Alternative electrophoretic methods such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), capillary electrophoresis (CE) and two-dimensional electrophoresis (2DE) have subsequently been used for fish speciation (Table 3). Recently, protein patterns of over 100 fish species were made available on an electronic database established by the FDA, known as the Regulatory Fish Encyclopedia (RFE). The RFE includes gel images and tabulated pI's that are intended to serve as references for species identification (FDA, 2009b). Nonetheless, the RFE mostly includes teleost species that are commercially important in the US, and its utility for fish identification in other countries is limited (Puyet & Bautista, 2010). High performance liquid chromatography (HPLC) has been used to generate species-specific profiles for raw fish samples (Armstrong & Leach, 1992; Sotelo *et al*., 1993). Further, the enzyme-linked immunosorbent assay (ELISA), an immunological method, uses antibodies that have been generated to detect target proteins in a given analyte. While the ELISA is promising for identification of certain fish, the required immunosera are not commercially available for routine analysis (Céspedes *et al*., 1998).

Although protein-based methods are of value for fish speciation in certain instances, they suffer from a number of disadvantages which make them unsuitable for routine sample analyses. Fish proteins may be denatured or degraded during food processing (heating, canning or drying), leading to alterations in the fingerprint patterns obtained by electrophoresis (Akasaki *et al*., 2006). Thus, protein-based methods are generally only applicable for species identification in raw fish, frozen fillets or mildly-treated products. The protein patterns derived from these methods may also differ depending on the age, developmental stage and tissue type of the sample analysed, leading to considerable intra-species variation (Martinez *et al*., 1991; Ferguson *et al.*, 1995). Additionally, due to the wide variety of fish species available, the accuracy of immunological methods may be compromised as a result of cross-reactivity with proteins of closely related species (Necidová *et al*., 2002). Therefore, authentication methods based on the analysis of nucleic acids (normally DNA) are considered preferable to those based on proteins, particularly when products are processed (Lockley & Bardsley, 2000a; Sebastio *et al*., 2001).

Table 3 Information obtained from various commonly utilised fish authentication techniques and the molecules targeted

Information	Molecule targeted	Analysis	Type of analysis	References
Species identification	Proteins	**Electrophoresis:**		
		SDS-PAGE	Fingerprints	Etienne *et al*., 2000; Corzo *et al*., 2005
		Isoelectric focusing (IEF)	Fingerprints	Rehbein *et al*., 1995, Piñeiro *et al*., 1998; Valenzuela *et al*., 1999; Etienne *et al*., 2001; Chen *et al*., 2003
		2D-electrophoresis	Fingerprints	Piñeiro *et al*., 1998; Valenzuela *et al*., 1999
		Capillary electrophoresis (CE)	Fingerprints	LeBlanc & LeBlanc, 1994; Gallardo *et al*., 1995
		Peptide mapping	Fingerprints	Rehbein, 2005
		Chromotograpnhic:		
		Liquid chromotography (LC)	Fingerprint and target recognition	Osman *et al*., 1987
		High Performance liquid chromatography (HPLC)		Armstrong & Leach, 1992
		Immunological methods:		
		Enzyme-linked immonosorbent assay (ELISA)	Target recognition	Taylor & Leighton Jones, 1992; Carrera *et al*., 1996; Carrera *et al*., 1997; Asensio & Lourdes, 2009
	DNA	**PCR-based:**		
		Restriction fragment length polymorphism (PCR-RFLP)	Fingerprint	Céspedes *et al.*, 1998; Quinteiro *et al*., 1998; Aranishi *et al*., 2005; Hsieh *et al.*, 2005; Hsieh *et al.*, 2007
		Single-strand conformation polymorphism (PCR-SSCP)	Fingerprint	Rehbein *et al*.,1997; 1999; Céspedes *et al*., 1999b; Asensio *et al*., 2001b; Comi *et al*., 2005
		Random amplified polymorphic DNA (RAPD)	Fingerprint	Dahle *et al*., 1997; Asensio *et al*., 2002; Jin *et al*., 2006
		Denaturing gradient gel electrophoresis (DGGE)	Fingerprint	Comi *et al*., 2005
		DNA sequencing	Target recognition	Hsieh *et al*., 2003; Jérôme *et al*., 2003; Marko *et al*., 2004; Ward *et al.*, 2005; Sevilla *et al*., 2007
		Species-specific PCR	Target recognition	Céspedes *et al.*, 1999a; Lin & Hwang, 2008
		Real-time PCR	Target recognition	Sotelo *et al*., 2003; Hird *et al*., 2005; Trotta *et al*., 2005
		PCR lab-on-chip	Target recognition	Dooley *et al*., 2005a; 2005b
	Lipids	Nuclear magnetic resonance (NMR): ^{13}C, ^{2}H, ^{1}H	Fingerprint	Martinez *et al*., 2005; Standal *et al*., 2010
Geographical origin	Lipids	Isotope ratio mass spectrometry (IRMS): $^{13}C/^{12}C$	Fingerprint	Martinez *et al*., 2005; Rezzi *et al*., 2007; Aursand *et al*., 2009
		Nuclear magnetic resonance (NMR): ^{13}C, ^{1}H, $^{16}O/^{18}O$	Fingerprint	
	Trace elements	Inductively coupled plasma mass spectrometry (ICP-MS)	Fingerprint	Martinez *et al*., 2005; Yamashita *et al*., 2006
Wild or farmed	Trace elements	ICP-MS	Fingerprint	Adey *et al*., 2009
	Lipids	^{13}C-NMR, ^{1}H-NMR, $^{13}C/^{12}C$-IRMS	Fingerprint	Aursand *et al*., 2000; Rezzi *et al*., 2007
	Proteins	$^{15}N/^{14}N$-IRMS	Fingerprint	Martinez *et al*., 2005
	Proteins	Protein patterns (proteomics)	Fingerprint and target recognition	Martinez *et al*., 2005

DNA-based methods for fish species identification

Although DNA was originally discovered by Friedrich Miescher in 1868 (Dahm, 2005), it was Erwin Chargaff that recognised in the early 1950s that the nucleotide composition of DNA differs between species (Chargaff, 1951). In the years to come, it was the latter discovery that led to DNA becoming the most powerful tool for species identification. The great variation afforded by the genetic code gives DNA analysis a high discriminatory power, allowing the differentiation between even very closely related species (Mackie, 1996; Woolfe & Primrose, 2004). DNA-based methods have several advantages for species identification over their protein-based counterparts. DNA is comparatively less sensitive to degradation by processing than proteins, remaining intact even at high temperatures (Lenstra, 2003). DNA-based methods can thus be used for the identification of not only fresh and frozen fish, but also processed, degraded and mixed fish products (Lockley & Bardsley, 2000a; Sebastio *et al*., 2001). Genomic DNA is also identical in almost all cell types of an individual, irrespective of its developmental stage, alleviating the need for standards for each analysed tissue type, as required for protein-based methods. Additionally, DNA sequence data can be more easily replicated and interpreted between laboratories than possible with data from protein-based techniques (Bossier, 1999; Sebastio *et al*., 2001, Martinez *et al*., 2005).

Suitability of DNA-based techniques for fish authentication

In order to be useful in promoting compliance with certain international labelling regulations, such as those set in the EU, the methods developed for fish authentication should ideally be able to provide information on the species, the country of origin and the technological method of production (wild or farmed) (EC, 2000b; 2001). It is likely that DNA-based methods will become the ‘gold standard’ approach for species identification, mainly due to their sensitivity, robustness and applicability to large-scale or routine sample analyses (Cocolin *et al*., 2000; Lockley & Bardsley, 2000a; Gil, 2007). Although it is beyond the scope of this thesis, it is worth noting that DNA based techniques are generally less suitable for the authentication of the geographical origin of biological samples and to distinguish wild from cultivated fish. While this is a developing field, spectroscopic techniques such as distribution of natural isotopes or trace element analysis, are preferred methods for geographical origin determination (Martinez *et al*., 2005) (Table 3). Methods based on trace element analyses, nuclear magnetic resonance and lipid profiling have proven adequate for the differentiation of wild and cultivated fish (Aursand *et al*., 2000; Moretti *et al*., 2003) (Table 3).

Extraction of DNA

All DNA-based techniques for fish species authentication rely on amplification using the polymerase chain reaction (PCR) and therefore require the extraction of DNA from the sample under study (Puyet & Bautista, 2010). The extraction of adequate quantities of pure DNA from a given sample is a primary determinant for the success of DNA-based methods (Aranishi, 2006; Lopera-Barrero *et al*., 2008). Ideally, the DNA extraction procedure should solubilise cellular components and simultaneously inactivate intracellular nucleases so that biologically active DNA is conserved. Cellular components (e.g. proteins, RNA and polysaccharides) or chemicals from the extraction method that are not removed from the DNA compromise the purity thereof and may inhibit subsequent PCR amplification (Merente *et al*., 1998; Di Pinto *et al*., 2007).

Traditional methods are generally adequate for the extraction of DNA from fish and fishery products (Puyet & Bautista, 2010). Such methods typically require a 5 - 50 mg sample of tissue to be excised from the inner portion of the specimen using a sterile scalpel or blade. The excised sample is minced and immersed in an extraction buffer, normally containing sodium dodecyl sulphate (SDS), ethylene diamine tetraacetic acid (EDTA) and proteinase K. This extraction buffer aids in the digestion of cellular components and the inactivation of endogenous DNase enzymes which would otherwise degrade the high molecular weight DNA (Saunders, 1999). In the case where DNA is extracted from fish fins or scales, urea is often incorporated into the extraction buffer to assist with the breakdown of these hard tissues (Asahida *et al.,* 1996). Once the cellular proteins and lipids are separated from the DNA, contaminating RNA is normally degraded by the addition of pure RNase, which is later removed with other contaminants by organic solvent (phenol and chloroform) extractions. The DNA is finally precipitated with absolute ethanol or isopropanol, collected by centrifugation and purified with 70% ethanol to remove any salts present in the DNA pellet (Rapley, 2003).

Phenol-chloroform extractions from fish have been shown to produce acceptable yields of DNA with relatively good quality for PCR and downstream applications (Asahida *et al.,* 1996; Wasko *et al*., 2003). However, besides being laborious due to the multiple additions of organic solvents, the safety of these methods is questionable as phenol and chloroform are hazardous reagents (Yue & Orban, 2001). More recently, numerous kits have been commercialised for the extraction and purification of DNA from animal tissues. Such commercial kits are convenient to use, incorporate safer reagents (avoiding the use of phenol and chloroform) and reportedly permit the extraction of higher quality DNA (Herman, 2001; Hajibabaei *et al*., 2005).

Choice of target DNA for PCR amplification

Most of the cellular information of eukaryotic organisms is located inside the nucleus (nuclear DNA; nDNA), while a smaller amount of DNA is contained within the mitochondria (mitochondrial DNA, mtDNA) (Hsieh *et al*., 2005). Advances in molecular techniques have permitted the direct analysis of the DNA sequences of both the nDNA and the mtDNA (Céspedes *et al*., 1998). However, there are a number of important factors that should be considered before a specific DNA segment is chosen for PCR amplification. For example, in order to be useful for species identification, it is essential that the region of the genome selected for amplification shows greater inter-species variation than intra-species variation (Hall & Nawroki, 1995; Hansen & Loeschcke, 1996). The length of the segment chosen must also be sufficiently long to permit the detection of differences in the DNA sequences of congeneric species, but also short enough so that the nucleotide sequence is determinable from a single loading of a standard DNA sequencing gel (Céspedes *et al*., 1998). In addition, since the creation of a data bank is a time-consuming and technically-demanding process, it is desirable to take advantage of those genes whose sequences have been determined for a diverse range of species (Bartlett & Davidson, 1992).

Several nDNA markers have been reported for fish species identification, including the 5S ribosomal DNA (rDNA) gene (Céspedes *et al*., 1999a), the alpha-actin gene (Watabe *et al*., 1995) and the growth hormone gene (Johansen *et al*., 1989). Among these nuclear targets, the 5S rRNA gene has been commonly exploited for species identification due to its remarkable structure, comprising a 120 base pair (bp) highly conserved coding sequence and tandem repeats of a variable non-transcribed spacer (NTS) region (Céspedes *et al*., 1999a; Asensio *et al*., 2001a). Since the length of the NTS is generally species specific, a simple PCR method is able to generate an electrophoretic pattern which allows for direct species identification without the need for DNA sequencing or the use of restriction enzymes (Sastri *et al*., 1992).

Analysis of mtDNA has found worldwide application for animal species identification, including seafood identification (Bartlett & Davidson, 1991; Bouchon *et al*., 1994). The mtDNA of animals is a small circular molecule, typically composed of 37 genes which code for two ribosomal RNAs (12S and 16S rRNAs), 22 transfer RNAs (tRNAs) and 13 messenger RNAs (mRNAs) coding for proteins. The control region or D-loop is the primary non-coding region of the mtDNA (Kleinsmith & Kish, 1995). Animal mtDNA possesses several characteristic features that have led to it being preferably used above nDNA for fish species identification:

1. The arrangement of mtDNA is very efficient and simple, with no complicated introns, repetitive sequences or pseudogenes (Gray, 1989; Sotelo *et al*., 1993);
2. The mtDNA is maternally inherited, not subject to the diversity-generating mechanisms associated with sexual re-assortment occurring in nDNA, and is thus conserved across long evolutionary distances (Lin *et al*., 1990; Rokas *et al*., 2003);
3. Several copies of mtDNA exist within a cell (versus only one or two copies of nDNA), facilitating analyses with degraded DNA or limited amounts of material (Trotta *et al*., 2005);
4. The mtDNA evolves more rapidly than the nDNA, making it easier to discriminate closely related species (Vawter & Brown, 1986; Zeviani *et al*., 1998); and
5. Complete mtDNA sequences of many fish species have been determined and these have been incorporated into integrated genomic databases, such as GenBank, the Barcode of Life Database (BOLD) and Fishtrace. These deposited mtDNA sequences serve as references and facilitate the design of PCR primers for identification (Zardoya *et al*., 1995; Miya *et al.*, 2003).

Polymerase chain reaction (PCR)

Since the discovery of the PCR (Mullis *et al*., 1986), the use of the technique has revolutionised molecular biology due to its ability to allow amplification and analysis of selected DNA fragments, even from very small quantities of starting material. In terms of fish species identification, this method has gained popularity due to its simplicity, sensitivity and specificity (Céspedes *et al*., 1998; Gil, 2007). The PCR uses a set of synthetic oligonucleotide primers, together with a thermostable DNA polymerase, each of four nucleotides (dNTPs), a buffer solution and a magnesium source, to direct the *in vitro* synthesis of millions of copies of the target DNA fragment using a repetitive thermal cycling process (Saiki *et al.*, 1988). The cycling process begins with an initial denaturation step, requiring heating at 94 - 95 ºC for *ca*. 60 s to separate the double-stranded DNA (dsDNA) helix. A reduction in temperature to 35 - 60 ºC for 30 - 120 s facilitates the annealing of the primers to the target regions of the single-stranded DNA (ssDNA) whose sequences are complimentary to theirs. During final extension at 72 ºC for 60 - 180 s, the DNA polymerase produces a complementary copy of the template DNA initiated by each primer. In each successive PCR cycle, the dsDNA produced from the previous cycle serves as a template for the production of a new dsDNA, giving rise to an exponential increase in the concentration of the target region (Rapley, 1998).

Design of PCR primers for species identification

Several parameters should be considered for the design of PCR primers. Generally, primers should have a length of at least 15 - 30 bp to be sufficiently specific and to anneal efficiently to the target DNA (Dieffenbach *et al*., 1993). The primers should ideally have a guanine-cytosine (GC) content of 40 - 60%, the sequences of which should not form primer-dimers or hairpin beacons during PCR amplification (Rapley, 1998). Additionally, primer pairs should be designed with a closely matching melting temperature (Tm), preferably with a difference of 5 ºC or less to promote optimum amplification (Rychlik *et al.*, 1990). In the case where limited sequence information is available for certain species, degenerate primers may be designed to incorporate alternative bases such as inosine at particular positions in the primer sequence (Knoth *et al.*, 1988). The size of the PCR fragment amplified by a primer pair is also important for species identification, especially when the analysed sample has been processed and the DNA is degraded into shorter sections. Nonetheless, even after extensive processing, DNA sequences of at least 100 - 200 bp in length are normally maintained that may be amplified by PCR (Quintero *et al*., 1998; Chapela *et al*., 2007).

Universal and species-specific PCR primers for species identification

PCR primers for fish species identification may be designed to be ‘universal’ for the detection of a large number of different species or ‘species-specific’ for the detection of only a specific target fish species. In general, universal primers are designed based on the sequences contained in evolutionary conserved regions of the animal genome, while species-specific primers are designed based on the non-conserved regions (Kitano *et al*., 2007; Laube, 2010). Most of the PCR methods developed for fish identification have relied on universal primers, since these can be applied for amplification when the precise DNA sequence of the target regions is unknown (Laube, 2010; Puyet & Bautista, 2010). Nonetheless such methods require the application of post-PCR methods to further identify and characterise the amplicon obtained (Rehbein, 2009). For the purpose of fish species identification, the mitochondrial sequences of the cytochrome *c* oxidase subunit I (COI), cytochrome *b* (cyt *b*), 12S and 16S rRNA genes and the control region are popular targets for PCR with universal primers (Kocher *et al*. 1989; Bartlett & Davidson, 1991; 1992; Ward *et al*., 2005; Di Finizio *et al*., 2007). The distance between the primer binding sites is normally selected to generate a PCR amplicon of a few hundred nucleotides for most fishery products (Rehbein, 2009), with the exception of thermally treated products in which smaller regions are generally targeted (Quintero *et al*., 1998). PCR applications using universal primers have led to the availability of detailed sequence information for many fish species, which make it possible to identify single base polymorphisms that can be exploited for the design of species-specific PCR primers for identification (Murgia *et al.*, 2002).

Unlike universal PCR methods, species-specific PCR methods are beneficial in that they do not necessitate further characterisation of the PCR amplicon obtained. The result of a species-specific PCR for species identification is usually positive or negative: either a PCR amplification product of the expected size is generated, in which case identity is confirmed, or one is not (Gil, 2007; Rastogi *et al*., 2007). As such, PCR with species-specific primers represents the fastest and most convenient method for fish species identification (Rehbein, 2009). Ideally, species-specific primers would be available for each possible species. However, with the vast number of fish species used as food, this has not yet been achieved (Martinez *et al*., 2005). In addition, due to the many closely related fish species that are utilised for human consumption (e.g. approximately 25 sturgeon species, 12 species from the *Merluccius* genus and more than 15 species from the Scombridae family) it is often difficult to design primers of adequate specificity that do not cross react with other species (Martinez *et al*., 2005; Rehbein, 2009). For instance, the species-specific primers used by Céspedes *et al.* (1999a) to identify flatfish were specific for sole (*Solea solea*), but those for Greenland halibut (*Reinhardtius hippoglossoides*) also reacted with other flatfish species. Similarly, primers designed to be specific for bluefin tuna (*Thunnus thynnus*) were found to also react with other *Thunnus* species (Rehbein, 2003). Consequently, many fish need to be tested with a set of primers before they can be deemed species-specific and appropriate controls should be included to preclude the possibility of false positive or false negative results being obtained (Edwards & Gibbs, 1994; Rehbein, 2009).

By combining multiple primer sets in the same PCR reaction, it is possible to produce PCR amplicons of varying sizes that are specific to different DNA sequences (Catanese *et al.*, 2010). This method, known as multiplex PCR, is highly suitable for species identification in mixed products where multiple types of fish may be present. However, optimisation of the annealing temperature for each primer set is vital for successful amplification and the amplified fragments should be sufficiently different to form distinct bands when visualised by gel electrophoresis (Gil, 2007). Multiplex PCR has the potential to reduce time and labour requirements in the laboratory and has been used successfully in numerous studies for fish identification (DeSalle & Birstein, 1996; Lockley & Bardsley, 2000b; Asensio *et al*., 2001a; Hsieh *et al.*, 2004).

Post-PCR visualisation

Regardless of whether universal or species-specific primers are used, conventional PCR methods require a post-run visualisation step to assess the PCR amplification products obtained. Most commonly, amplified DNA fragments are separated according to their size by agarose gel electrophoresis and are visualised by staining with an intercalating dye, such as ethidium bromide (Walker, 2003). Apart from when species-specific primers are used, gel electrophoresis only confirms the presence of target fragments, but does not provide species information. Further analysis is thus required to characterise the amplicons based on their unique nucleotide profile. Fingerprinting methods or direct DNA sequencing are commonly applied for the analysis of PCR products to obtain the information needed to identify the samples (Rehbein, 2003).

PCR product analysis (fingerprinting)

A number of DNA fingerprinting techniques have been applied for fish species identification, mainly because they are more rapid, less expensive and less technically demanding than DNA sequencing approaches (Rasmussen & Morrissey, 2009). Fingerprint-based identifications are beneficial for confirmation of a species when an unknown sample can be simultaneously compared with a suitable reference sample (Martinez *et al.*, 2001). However, in the absence of control samples from all potential substitution species, they may be limited in their capacity to identify unknown species in adulterated fish products (Puyet & Bautista, 2010). Furthermore, species identifications based on fingerprints may be compromised when products contain mixtures of specimens (Martinez & Yman, 1998) or when DNA is degraded due to processing (Rehbein, 2009). Of the PCR-based fingerprinting methods developed for species identification, PCR restriction fragment length polymorphism (PCR-RFLP), PCR single-strand conformational polymorphism (PCR-SSCP) and random amplified polymorphic DNA (RAPD) have been most commonly applied for fish authentication (Gil, 2007).

Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP)

PCR-RFLP is the most simple and commonly used fingerprinting methodology used for the routine identification of fish species (Aranishi *et al*., 2005; Hsieh *et al*., 2007). The technique requires the selection of one or more restriction endonucleases that cleave the amplified DNA at defined nucleotide sites to generate a unique electrophoretic fingerprint pattern that may be utilised for species identification (Rehbein, 2003). PCR-RFLP methods, based mostly on fragments of the mitochondrial cyt *b* gene, have been used with varying success for the discrimination of flatfish (Céspedes *et al.*, 1998; Comesaña *et al.*, 2003), billfish (Hsieh *et al.*, 2005; 2007), gadoid fish (Hold *et al*., 2001; Calo-Mata *et al*., 2003; Aranishi *et al*., 2005; Comi *et al*., 2005; Dooley *et al*., 2005a; Akasaki *et al*., 2006) and canned tuna (Ram *et al*., 1996; Quinteiro *et al*., 1998; Pardo & Pérez-Villareal, 2004). A similar approach targeting the control region was employed to identify 11 *Merluccius* species (Quinteiro *et al*., 2001) and Aranishi *et al*. (2005) used PCR-RFLP on amplicons of the 5S rDNA NTS region to discriminate mackerel species.

PCR-RFLP, nonetheless, suffers from a number of limitations in terms of its application to fish species authentication. Incomplete DNA digestion by restriction endonucleases may lead to sub-optimal fingerprint patterns and intra-specific variation may potentially delete or create additional restriction sites (Lockley & Bardsley, 2000a). Further, since DNA is usually severely degraded during food processing methods such as canning (Akasaki *et al*. 2006), PCR-RFLP analysis of such products generally targets relatively short DNA regions (Meyer *et al*., 1995) which may have limited exploitable restriction sites for fingerprinting.

Polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP)

Authentication using PCR-SSCP takes advantage of the quality of single stranded DNA and the difference in its electrophoretic mobility as a means of detecting sequence variation (Orita *et al*., 1989). In the case of conventional dsDNA gel electrophoresis, mobility is mainly dependent on the size and length of the strand, while the particular nucleotide sequence has little impact in this regard. After denaturation, however, very subtle differences in the ssDNA sequences become apparent due to the unique 3-dimensional folding that the molecule undergoes based on its primary structure (Scoggan & Bulman, 2003). Consequently, even single base variations in the conformation of two ssDNA strands with different sequences results in variation in their electrophoretic mobility during native gel electrophoresis even if the number of nucleotides are the same (Hayashi, 1991; Oohara, 1997). Unlike PCR-RFLP, analysis with SSCP is beneficial in that it is able to detect polymorphisms at multiple sites in the DNA fragment (Orita *et al*., 1989) and thus even closely related species may be discriminated. Additionally, SSCP is suited for the analysis of short DNA strands (less than 300 bp) and permits species identification of degraded DNA (Rehbein *et al*., 1999).

In spite of the benefits of SSCP, the technique may also be adversely influenced by certain test parameters, such as the type of denaturing solution utilised, electrophoresis temperature, gel and buffer concentrations, as well as the addition of various compounds to the gel matrix (Fujita & Silver, 1994; Scoggan & Bulman, 2003). For reproducible DNA patterns to be obtained by SSCP, analysis must therefore be performed under stringently controlled conditions (Hayashi, 1991). PCR-SSCP has been successfully used for the identification of tuna (Rehbein *et al*., 1999; Weder, *et al*., 2004), cod (Comi *et al*., 2005), flatfish (Céspedes *et al*., 1999b), Nile perch, grouper, wreck fish (Asensio *et al*., 2001b), sturgeon, salmon and trout (Rehbein *et al*.,1997).

Random amplified polymorphic DNA (RAPD)

RAPD analysis involves PCR amplification with short primers, generally 10 bp in length, which anneal at low temperatures (35 - 40 °C) to various arbitrary sites in the DNA to generate a spectrum of DNA fragments after electrophoresis of the resulting PCR products (Puyet & Bautista, 2010; Rehbein, 2009). RAPD analysis is cheaper, faster and simpler than most other DNA-based authentication methods, not requiring prior knowledge of the genetic make-up of the targeted fish species (Williams *et al*., 1990; Partis & Wells, 1996). Nonetheless, the major disadvantage of the method lies in the poor reproducibility of the generated results (Welsh & McClelland, 1990). The technique is difficult to standardise and the results depend highly on the quality of the DNA being amplified, as well as the PCR and electrophoresis conditions. As with PCR-RFLP, analysis of mixed species products or heat processed products in which DNA is degraded may not be feasible with RAPD analysis (Martinez & Yman, 1998). RAPD has, however, been reported for the identification of several fish products, such as salmonids (Yamazaki *et al*., 2005; Jin *et al*., 2006), tilapia (Bardakci & Skibinski, 1994), Nile perch, grouper, wreck fish (Asensio *et al*., 2002) and shad (Dahle *et al*., 1997).

New and emerging DNA-based technologies for fish species identification

Real-time PCR

In contrast to conventional qualitative PCR, real-time PCR (RT-PCR) offers a quantitative approach to food authentication (López-Calleja *et al*., 2007). Quantitative monitoring proves valuable for assessing fish products containing multiple species, levels of species adulteration and even for the detection of minute amounts of contaminating fish residues in foodstuffs which have the ability to provoke allergic reactions in sensitive individuals (Rapley, 2003; Holzhauser *et al*., 2006). Although some RT-PCR applications make use of fluorescent dyes such as Sybr Green, fluorophore methods such as the TaqMan assay are very promising for this purpose.

The TaqMan assay utilises an oligonucleotide probe, labelled with a fluorescent reporter dye on the 5' end and a quencher dye at the 3' end, which is designed to anneal to a specific position between the two primers (Woolfe & Primrose, 2004). During amplification, the 5' to 3' activity of the *Taq* DNA polymerase cleaves and releases the reporter molecule. Cleavage of the probe leads to an increase in fluorescence, which is proportional to the amount of template DNA that is amplified (Holland *et al*., 1991). DNA is quantified by determining the threshold cycle (Ct), which is the cycle of the PCR at which the fluorescence reaches a predetermined value above the background (Woolfe & Primrose, 2004). Fluorescence detection by RT-PCR is beneficial in that it eliminates the requirement for post-PCR gel electrophoresis needed with conventional PCR, thus promoting the automation and large scale processing abilities of the technique (Gil, 2007). The RT-PCR TaqMan assay has been effectively used for the identification and quantification of cod (Sotelo *et al*., 2003), haddock (Hird *et al*., 2005), as well as tuna species (López & Pardo, 2005). In addition, Trotta *et al*. (2005) used this method to identify grouper fillets and commonly substituted species.

PCR lab-on-a-chip

During the last decade, much interest has focused on the performance of laboratory operations on a miniaturised scale using lab-on-a-chip devices of only a few square centimetres in size (De Mello, 2001). Based on this principle, high efficiency micro-fluidic PCR devices have been developed (Zhang *et al*., 2006; Zhang & Zing, 2007). With such systems, extremely small sample volumes (picolitre scale) can be processed with a high degree of control. This not only allows for a reduction in the reagent costs and chemical waste associated with analysis, but also results in considerable time savings due to the smaller thermal capacity and the greater rates of heat transfer between the sample and the temperature-controlling components (Roper *et al*., 2005, Daw & Finkelstein, 2006). PCR lab-on-a-chip has recently been applied for fish species authentication purposes, however, reports on such applications are currently limited. Dooley *et al*. (2005a) used this technology to discriminate admixtures of 5% salmon DNA in trout DNA and reported that the method provided improved resolution and sensitivity compared to PCR-RFLP analysis. The same authors employed this method for the detection of 10 white fish species in UK food products (Dooley *et al*., 2005b). It is likely that as the development of PCR chips continues, these will be used in various applications, including fish authentication (Zhang & Zing, 2007; Dooley *et al*., 2005a).

Commercial PCR kits for fish species differentiation

Advances in PCR-based technologies have led to the development of a number of commercial kits for fish identification, which generally include all the reagents, controls and accessories required for testing. A multiplex PCR kit has been launched by Tepnel Biosystems (www.tepnel.com) that allows for the qualitative detection of eight fish species (cod, coley, hake, haddock, pollock, trout, salmon and whiting). The Spanish company Biotools (www.biotools.eu) has commercialised different Biofish kits (e.g. a cod kit and a salmon kit) based on PCR-RFLP analysis, as well as PCR-sequencing kits for the species-level identification of flatfish, hake, tuna and sardine. The fishID kit from Bionostra (www.bionostra.com) allows for identification of more than 200 fish species based on PCR amplification of specific mtDNA sequences. In addition, Biomerieux (www.biomeriex.com) developed the first high-density DNA Chip for animal species identification (GeneChip®), which allows the identification of many fish species. The simple, rapid nature of these diagnostic kits will likely lead to them being increasingly used by regulatory agencies for the detection of fish species adulteration (Gil, 2007).

DNA sequencing

DNA sequencing, a technique that determines the precise order of nucleotide bases (adenine, cytosine, guanine and thymine) in amplified DNA fragments, is recognised as the most informative and accurate method for species identification (Unseld *et al*., 1995). The concept of combining PCR and DNA sequencing for animal species identification was introduced by Bartlett and Davidson (1992) under the name FINS (Forensically Informative Nucleotide Sequencing). FINS involves the comparison of an unknown sequence with a pool of reference sequences by using of genetic distance measurements (Forrest & Carnegie, 1994) and phylogenetic tree-building that clusters the unknown sequence with those to which it is most closely related (Rehbein, 2009).

A simpler manner of making a species identification is by comparing an unknown sequence with reference nucleotide sequences which have been deposited into web-accessible genetic databases such as GenBank (www.ncbi.nlm.nih.gov). The most likely identity of a specimen is established by using an index of similarity between two or more sequences, calculated by means of the BLAST (Basic Local Alignment Search Tool) algorithm (Brodmann *et al*., 2001). Apart from GenBank, fish DNA sequences can also be obtained from other online databases, such as BOLD (Barcode of Life Database) (www.boldsystems.org) and Fishtrace (www.fishtrace.org). The use of these databases allows for species identification without the requirement for reference material, provided that the generated sequence is available in the database. To maximise the chance of making a species identification in this manner, it is essential that a region is chosen for sequencing for which there is a large number of entries in such databases, such as the cyt *b* or COI gene (Teletchea, 2009). However, the sequences of some closely related fish species may be almost identical in certain genetic regions, which may necessitate the sequencing of more than one DNA fragment for an accurate species identification to be made (Puyet & Bautista, 2010). Additionally, the lack of deposited sequences for some commercial fish in genetic databases precludes the identification of such species by DNA sequencing (Schander & Willassen, 2005; Von der Heyden *et al*., 2010).

DNA sequencing is not a simple technique, requiring sophisticated laboratory equipment and considerable skill on the part of the analyst. Additionally, the technique cannot be used directly for the analysis of samples containing mixed species (Rehbein, 2009; Puyet & Bautista, 2010). Despite these limitations, information obtained from DNA sequencing, particularly that from mtDNA, has been successfully used to identify species of sardine (Jérôme *et al*., 2003), grey mullet (Murgia *et al*., 2002), tuna and billfish (Richardson *et al.*, 2007) and many other teleosts (Sevilla *et al.*, 2007). In addition, mtDNA sequencing was used to evaluate the extent of mislabelling of red snapper (Marko *et al*., 2004) and to identify fish species in adulterated dried mullet roe (Hsieh *et al.*, 2003). Sequencing of the COI gene has also been used for the identification of marine fish from Australia (Ward *et al.*, 2005), Canada pacific (Steinke *et al*., 2009) and the Scotia Sea (Rock *et al*., 2008) for DNA barcoding purposes.

DNA barcoding

The use of DNA barcoding as a new tool for animal species identification has recently gained much worldwide attention and promotion in numerous peer-reviewed scientific publications (Hebert *et al*., 2003a; Ward *et al*., 2005). The concept of DNA barcoding is based on the premise that the sequence diversity contained in short standardised regions of the genome is able to serve as a unique ‘signature’ (or DNA barcode) for each species (Ratnasingham & Hebert, 2007). The DNA region nominated and widely adopted for DNA barcoding is a *ca.* 650 bp region of the mtDNA COI gene, which has now been validated as a diagnostic marker for the identification of a diverse range of animal species (Dawnay *et al*., 2007; Ward *et al*., 2009). It has been reported that this sequence provides adequate identification labels in terms of nucleotide positions to discriminate even congeneric fish species, in spite of only a 2% sequence divergence being found in 98% of these species (Hebert *et al*., 2003a; 2003b; Ward *et al*., 2005).

In order for the barcoding approach to be used on a large scale, the generation of DNA barcodes for each species is needed to provide a key for identifying unknown specimens (Rastogi *et al*., 2007; Ward *et al.*, 2009). Realising that reliable species identification would require comprehensive management of these multitudes of genetic data, Hebert *et al.* (2003a) proposed the compilation of a public library of DNA barcodes that could be linked to named species. Early success achieved with the barcoding of fish species (Savolainen *et al*., 2005; Ward *et al*., 2005) led to the establishment of the Fish Barcode of Life Initiative (FISH-BOL) (www.fishbol.org) in 2005. FISH-BOL is an international collaborative research campaign that aims to assemble a standardised reference repository of barcode sequences for all fish (more than 30 000 species worldwide) obtained from voucher specimens with authoritative taxonomic identifications (Ward *et al*., 2009). Barcode sequences derived from whole fish, fillets, fins, juveniles, eggs or larvae from any specimen may be matched with deposited sequences in BOLD. To date (April 2011), more than 8 200 fish species have been COI barcoded.

Although GenBank includes multitudes of fish DNA sequences, most past records have lacked an explicit connection to vouchers. This is problematic as, if errors in the submissions are discovered, specimen misidentifications are difficult to resolve in the absence of the original material (Ruedas *et al*., 2000; Harris, 2003; Pleijel *et al*., 2008). The Consortium for the Barcode of Life (CBOL) has been tasked with ensuring stringent data standards for DNA barcode submissions to ensure that they are fit for molecular diagnostic applications (Lorenz *et al*., 2005). The submission of barcode data to BOLD must be accompanied by a valid species name, voucher designation (including where the voucher is stored), details on the origin, collection date, collector and identifier of the specimen, and the PCR primers used to generate the sequence (Ward *et al*., 2009). Although DNA barcoding for fish species identification has not been officially accepted by regulatory bodies, the FDA is considering the method as a replacement for protein IEF for this purpose (Yancy *et al*., 2007).

In spite of its great promise for species identification, the emergence of DNA barcoding has been met with mixed reaction from certain scientific circles (Moritz & Cicero, 2004). Such reactions have ranged from enthusiasm (Stoeckle, 2003; Janzen, 2004) to condemnation (Seberg *et al.*, 2003; Will & Rubinoff, 2004). Most controversy has been in response to the notion that the COI region can be exclusively utilised as an all-purpose diagnostic tool for the identification of all species (Tautz *et al*., 2002; 2003; Blaxter, 2004). Some researchers, averse to the idea that this technique could replace morphological identification, have argued that barcoding is a gross oversimplification of the science of taxonomy (Will & Rubinoff, 2004; Ebach & Holdredge, 2005). It is well established that several processes such as inconsistent mutation rates, introgressive hybridization, heteroplasmy, pseudogene ontogenesis and the retention of ancestral polymorphisms may pose problems for resolving species boundaries using mtDNA sequences (Zhang & Hewitt, 1996; Funk & Omland, 2003; Rubinoff *et al*., 2006; Hubert *et al*., 2007). Concerns have been raised on whether recently diverged species will be distinguishable from their COI sequences (Dasmahapatra & Mallet, 2006). Although uncommon, there are undoubtedly certain species that cannot be differentiated by barcoding (Ward *et al*., 2009). The skates *Bathyraja lindbergi* and *Bathyraja maculate* (Spies *et al.*, 2006) and sting rays *Urolophus sufflavus* and *Urolophus cruciatus* (Ward & Holmes, 2007; Ward *et al*., 2008) are examples of such cases, as are certain freshwater species which share barcode haplotypes (Hubert *et al*., 2008).

The efficiency of barcoding can reportedly be enhanced by the use of alternative DNA sequences with different genomic locations and evolutionary rates. For example, the barcoding approaches proposed by FishTrace combine the use of the complete mtDNA cyt *b* gene sequence (1141 bp) and a partial fragment of the nDNA rhodopsin gene (460 bp) (Sevilla *et al*., 2007). The use of more than one gene for barcoding allows for cross-checking of species identifications, as each sequence is independently validated and phylogentically analysed (Puyet & Bautista, 2010). In addition, simultaneous analysis of the rapidly evolving mtDNA control region may be beneficial when species resolution is unachievable by COI barcoding alone (Ward *et al*., 2009).

Conclusions

Globally, the human appetite for seafood has escalated. Increasing demand for fishery products has led to extensive overfishing and the depletion of many marine fish populations. Compounding resource scarcity and the potential for greater profits have encouraged the incorrect labelling and fraudulent trading of fish commodities on world markets, the results of which have economic, environmental and health impacts. The increasing processing of fish products and the international trade in fish commodities have made fish identification by visual inspection problematic and have only exacerbated the incidence of mislabelling. The renaming and mislabelling of fish in

South Africa and worldwide is therefore not just an indication of cheating, but it is essentially an indication that both local and global fisheries are in distress (Jacquet & Pauly, 2008a). In response to this situation, many countries have passed legislation governing the labelling of fishery products. However, such legislation has proved to be incomplete in certain instances, or its enforcement has been too weak to promote fair trade in the industry. Consequently, there is now a greater need than ever before for food control authorities to pursue the development and implementation of analytical testing methods to authenticate a wide variety of fish at the species level.

Of the analytical methods available, protein-based methods will probably become less valuable for species identification with the increasing number of processed products that need to be authenticated. DNA-based methods based on PCR amplification and DNA sequencing, on the other hand, will likely become the gold standard approach for the identification of fish species because they can be applied to any body fragment of both raw and processed fish and because they are rapid, sensitive and specific. Taking steps to better control fish trade by authentication testing will not only be an important step towards the prevention of fraud, but also an important step towards promoting sustainability of marine resource. Without such steps, it appears increasingly likely that many fish species that humans know and enjoy at this time, will only be seen under the extinct list in textbooks by future generations.

References

Adey, E.A., Black, K.D., Sawyer, T., Shimmield, T.M. & Trueman, C.N. (2009). Scale microchemistry as a tool to investigate the origin of wild and farmed *Salmo salar*. *Marine Ecology Progress Series*, **390**, 225-235.

Akasaki, T., Yanagimoto, T., Yamakami, K., Tomonaga, H. & Sato, S. (2006). Species identification and PCR-RFLP analysis of cytochrome *b* gene in cod fish (order *Gadiformes*) products. *Journal of Food Science*, **71**, 190-195.

Alexander, J., Autrup, H., Bard, D., Carere, A., Costa, L.G., Cravedi, J.P., Domenico, A.D., Fanelli, R., Fink-Gremmels, J., Gilbert, J., Grandjean, P., Johansson, N., Oskarsson, A., Renwick, A., Ruprich, J., Schlatter, J., Schoeters, G., Schrenk, D., van Leeuwen, R. & Verger, P. (2004). Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to the toxicity of fishery products belonging to the family of *Gempylidae*. *European Food Safety Authority Journal*, **92**, 1-5.

Alongi, D.M. (2002). Present state and future of the world's mangrove forests. *Environmental Conservation*, **29**, 331-349.

AOAC (Association of Official Analytical Chemists) (1995). AOAC Official Method 980.16: Identification of fish species. In: *Official Methods of Analysis*, Vol. II, 16th ed., 35.1.41. (edited by P. Cuniff). Maryland, USA: AOAC International.

Aranishi, F. (2006). Single fish egg DNA extraction for PCR amplification. *Conservation Genetics*, **7**, 153-156.

Aranishi, F., Okimoto, T. & Izumi, S. (2005). Identification of gadoid species (Pisces, *Gadidae*) by PCR-RFLP analysis. *Journal of Applied Genetics*, **46**, 69-73.

Armstrong, S.G. & Leach, D.N. (1992). The use of HPLC protein profiles in fish species identification. *Food Chemistry*, **44**, 147-155.

Asahida, T., Kobayashi, T., Saitoh, K. & Nakayama, I. (1996). Tissue preservation and total DNA extraction from fish stored at ambient temperature using buffers containing high concentration of urea. *Fish Science*, **62**, 727-730.

Asensio, L., González, I., Fernández, A., Céspedes, A., Rodríguez, M.A., Hernández, P.E., García, T. & Martín, R. (2001a). Identification of Nile perch (*Lates niloticus*), grouper (*Epinephelus guaza*), and wreck fish (*Polyprion americanus*) fillets by PCR amplification of the 5S rDNA gene. *Journal of the AOAC International*, **84**, 777-781.

Asensio, L., González, I., Fernández, A., Rodríguez, M.A., Hernández, P.E., García, T. & Martín, R. (2001b). PCR-SSCP: a simple method for the authentication of grouper (*Epinephelus guaza*), wreck fish (*Polyprion americanus*) and Nile perch (*Lates niloticus*) fillets. *Journal of Agricultural and Food Chemistry*, **49**, 1720-1723.

Asensio, L., González, I., Fernández, A., Rodríguez, M.A., Lobo, E., Hernández, P.E., García, T. & Martín, R. (2002). Application of random amplified polymorphic DNA (RAPD) analysis for identification of grouper (*Epinephelus guaza*), wreck fish (*Polyprion americanus*), and Nile perch (*Lates niloticus*) fillets. *Journal of Food Protection*, **65**, 432-435.

Asensio, L. & Lourdes, S. (2009). Rapid identification of grouper and wreck fish meals by ELISA: a field study in restaurants. *International Journal of Food Science & Technology*, **44**, 1585-1589.

Aursand, M., Mabon, F. & Martin, G.J. (2000). Characterization of farmed and wild salmon *Salmo salar* by a combined use of compositional and isotopic analyses. *Journal of the American Oil Chemists Society*, **77**, 659-666.

Aursand, M., Standal, I.B., Pra, A., McEvoy, L., Irvine, J. & Axelson, D.E. (2009). ^{13}C NMR pattern recognition techniques for the classification of Atlantic salmon (*Salmo salar* L.) according to their wild, farmed, and geographical origin. *Journal of Agricultural and Food Chemistry*, **57**, 3444-3451.

Bardakci, F. & Skibinski, D.O.F. (1994). Application of the RAPD technique in tilapia fish: species and subspecies identification. *Heredity*, **73**, 117-123.

Bartlett, S.E. & Davidson, W.S. (1991). Identification of *Thunnus* tuna species by the polymerase chain reaction and direct sequence analysis of their mitochondrial cytochrome *b* genes. *Journal of Fisheries and Aquatic Science*, **48**, 309-317.

Bartlett, S.E. & Davidson, W.S. (1992). FINS forensically informative nucleotide sequencing – a procedure for identifying the animal origin of biological specimens. *Biotechniques*, **12**, 408-411.

Béné, C. (2008). Global change in African fish trade: engine of development or threat to local food security? In: *OECD Food, Agriculture and Fisheries Working Paper No. 10.* Pp. 1-19. Malaysia: OECD publishing.

Béné, C. & Heck, S. (2005). Fish and food security in Africa. *NAGA WorldFish Center Quarterly*, **28**, 8-13.

Berman, P., Harley, E.H. & Spark, A.A. (1981). Keriorrhoea - the passage of oil per rectum - after ingestion of marine wax esters. *South African Medical Journal*, **59**, 791-792.

Blaxter, M.L. (2004). The promise of a DNA taxonomy. *Philosophical Transactions of the Royal Society B*, **359**, 669-679.

Bossier, P. (1999). Authentication of seafood products by DNA patterns. *Journal of Food Science*, **64**, 189-193.

Bostock, T. & Herdson, D. (1985). La pesca y utilizacion del tiburon el el Ecuador. *Insitute Nacion de Pesca Boletin Cientifico y Tecnico*, **8**, 21-28 (As cited by Jacquet & Pauly, 2007).

Bouchon, D., Souty-Grosset, C. & Raimond, R. (1994). Mitochondrial DNA variation and markers of species identity in two panaeid shrimp species: *Penaeus monodon Fabricius* and *P. japonicus* Bate. *Aquaculture,* **127**, 131-144.

Branch, T. (2008). Not all fisheries will be collapsed in 2048. *Marine Policy*, **32**, 38-39.

Branch, G. & Clark, B. (2006). Fish stocks and their management: the changing face of fisheries in South Africa. *Marine Policy*, **30**, 3-17.

Brodmann, P.D., Nicholas, G., Schaltenbrand, P. & Ilg, E.C. (2001). Identifying unknown game species: experience with nucleotide sequencing of the mitochondrial cytochrome *b* gene and a subsequent basic local alignment search tool search. *European Food Research and Technology*, **212**, 491-496.

Brunner, E. (2006). Oily fish and omega-3 fat supplements. *British Medical Journal*, **332**, 739-740.

Brunner, E., Jones, P.J.S., Friel, S. & Bartley, M. (2009). Fish, human health and marine ecosystem health: policies in collision. *International Journal of Epidemiology*, **38**, 93-100.

Buck, E.H. (2009). Seafood marketing: combating fraud and deception. [Internet document]. URL www.nationalaglawcenter.org/assets/crs/RL34124.pdf. 20 October 2010.

Burger, J. & Gochfeld, M. (2004). Mercury in canned tuna: white versus light and temporal variation. *Environmental Research*, **96**, 239-249.

Burros, M. (1992). Pollack or cod? Fish or foul? FDA take a closer look. *New York Times*, September 2, 1992.

Burros, M. (2005). Stores say wild salmon, but tests say farm bred. *New York Times*, April 10, 2005.

Calo-Mata, P., Sotelo, C.G., Pérez-Martin, R.I., Rehbein, H., Hold, G.L., Russell, V.J., Pryde, S., Quinteiro, J., Rey-Méndez, M., Rosa, C. & Santos, A.T. (2003). Identification of gadoid fish species using DNA-based techniques. *European Food Research and Technology*, **217**, 259-264.

Carrera, E., Martín, R., García, T., González, I., Sanz, B. & Hernández, P.E. (1996). Development of an enzyme-linked immunosorbent assay for the identification of smoked salmon (*Salmo salar*), trout (*Oncorhynchus mykiss*) and bream (*Brama raii*). *Journal of Food Protection*, **59**, 521-524.

Carrera, E., García, T., Céspedes, A., González, I., Sanz, B., Hernández, P.E. & Martín, R. (1997). Immunostick colorimetric ELISA assay for the identification of smoked salmon (*Salmo salar*), trout (*Oncorhynchus mykiss*) and bream (*Brama raii*). *Journal of the Science of Food and Agriculture*, **74**, 547-550.

Catanese, G., Manchado, M., Fernández-Trujillo, A. & Infante, C. (2010). A multiplex-PCR assay for the authentication of mackerels of the genus *Scomber* in processed fish products. *Food Chemistry*, **122**, 319-326.

Céspedes, A., García, T., Carrera, E., González, I., Sanz, B., Hernández, P.E. & Martín, R. (1998). Identification of flatfish species using polymerase chain reaction (PCR) amplification and restriction analysis of the cytochrome *b* gene. *Journal of Food Science*, **63**, 206-209.

Céspedes, A., García, T., Carrera, E., González, I., Fernández, A., Hernández, P.E. & Martín, R. (1999a). Identification of sole (*Solea solea*) and Greenland halibut (*Reinhardtius hippoglossoides*) by PCR amplification of the 5S rDNA gene. *Journal of Agricultural and Food Chemistry*, **47**, 1046-1050.

Céspedes, A., García, T., Carrera, E., González, I., Fernández, A., Hernández, P.E. & Martin, R. (1999b). Application of polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) to identification of flatfish species. *Journal of the AOAC International*, **82**, 903-907.

Chapela, M.J., Sotelo, C.J., Pérez-Martín, R.I., Pardo, M.Á., Pérez-Villareal, B., Gilardi, P. & Riese, J. (2007). Comparison of DNA extraction methods from muscle of canned tuna for species identification. *Food Control*, **18**, 1211-1215.

Chargaff, E. (1951). Structure and function of nucleic acid as cell constituent. *Federation Proceedings*, **10**, 654-659.

Chen, T.Y., Shiau, C.Y., Moguchi, T., Wei, C. & Hwang, D.F. (2003). Identification of puffer fish species by native isoelectric focusing technique. *Food Chemistry*, **83**, 475-479.

Chong, W. (2007). Sale of oilfish to be curbed. *The Standard*, January 26, 2007.

Clover, C. (2006). *The End of the Line: How Overfishing is Changing the World and What We Eat*. Pp. 1-386. New York, USA: The New Press.

Cocolin, L., Agaro, E.D., Manzano, M., Lanari, D. & Comi, G. (2000). Rapid PCR-RFLP method for the identification of marine fish fillets (seabass, seabream, umbrine, and dentex). *Journal of Food Science*, **65**, 1315-1317.

Codex Alimentarius (1985). Codex general standard for the labelling of prepackaged foods. In: *CODEX STAN 1-1985*. Pp. 1-46. Rome, Italy: FAO.

Cohen, N.J., Deeds, J.R., Wong, E.S., Hanner, R.H., Yancy, H.F., White, K.D., Thompson, T.M., Wahl, M., Pham, T.D., Guichard, F.M., Huh, I., Austin, C., Dizikes, G. & Gerber, S.I. (2009). Public health response to puffer fish (tetrodotoxin) poisoning from mislabeled product. *Journal of Food Protection*, **72**, 810-817.

Comesaña, A.S., Abella, P. & Sanjuán, A. (2003). Identification of flatfish by PCR-RFLP. *Journal of the Science of Food and Agriculture*, **83**, 752-759.

Comi, G., Iacumin, L., Rantsiou, K., Cantoni, C. & Cocolin, L. (2005). Molecular methods for the differentiation of species used in production of cod-fish can detect commercial frauds. *Food Control*, **16**, 37-42.

Constance, D.H. & Bonanno, A. (2000). Regulating the global fisheries: the World Wildlife Fund, Unilever, and the Marine Stewardship Council. *Agriculture and Human Values*, **17**, 125-139.

Corzo, J., Riol-Cimas, J.M. & Meléndez-Hevia, E. (2005). Fish species identification by electrophoresis of muscle proteins in sodium dodecyl sulfate-containing polyacrylamide slab gels. *Electrophoresis*, **5**, 168-170.

Cox, W.M. & Reid, E.E. (1932). The chemical composition of oil of *Ruvettus pretiosus*, the ‘‘castor oil fish”. *Journal of the American Chemical Society*, **54**, 220-229.

Cummins, A. (2004). The Marine Stewardship Council: a multi-stakeholder approach to sustainable fishing. *Corporate Social Responsibility and Environmental Management*, **11**, 85-94.

Dahle, G., Rahman, M. & Eriksen, A.G. (1997). RAPD fingerprinting used for discriminating among three populations of Hilsa shad (*Tenualosa ilisha*). *Fisheries Science*, **32**, 263-269.

Dahm, R. (2005). Friedrich Miescher and the discovery of DNA. *Developmental Biology*, **278**, 274-288.

Dasmahapatra, K.K. & Mallet, J. (2006). DNA barcodes: recent successes and future prospects. *Heredity*, **97**, 254-255.

Daw, R. & Finkelstein, J. (2006). Lab on a chip. *Nature*, **442**, 367.

Dawnay, N., Ogden, R., McEwing, R., Carvalho, G.R. & Thorpe, R.S. (2007). Validation of the barcoding gene CO1 for use in forensic genetic species identification. *Forensic Science International*, **173**, 1-6.

DEAT (Department of Environmental Affairs & Tourism) (2010). State of the environment, Republic of South Africa: demersal fisheries. [Internet document]. URL http://soer.deat.gov.za/themes.aspx?m=231. 18 April 2010.

De Graaf, G.J. & Xuan, T.T. (1998). Extensive shrimp farming, mangrove clearance and marine fisheries in the southern provinces of Vietnam. *Mangroves and Salt Marshes*, **2**, 159-166.

Delgado, C.L., Wada, N., Rosegrant, M.W., Meijer, S. & Ahmed, M. (2003). *The Future of Fish: Issues and Trends to 2020*. Pp. 1-5. Washington DC, USA: International Food Policy Research Institute and World Fish Center.

De Mello, A.J. (2001). DNA amplification: does ‘small’ really mean ‘efficient’? *Lab on a Chip*, **1**, 24N-29N.

Denn, R. (2003). PCC’s ‘premium fish’ was wrongly labeled. *Seattle Post-Intelligencer*, October 24, 2003.

DeSalle, R. & Birstein, V.J. (1996). PCR identification of black caviar. *Nature*, **381**, 197-198.

De Walt, B.R., Vergne, P. & Harding, M. (1996). Shrimp aquaculture development and the environment: people, mangroves and fisheries on the Gulf of Fonseca, Honduras. *World Development*, **24**, 1193-1208.

Dieffenbach, C.W., Lowe, T.M. & Dveksler, G.S. (1993). General concepts for PCR primer design. *Genome Research*, **3**, S30-S37.

Di Finizio, A., Guerriero, G., Russo, G.L. & Ciarcia, G. (2007). Identification of gadoid species (Pisces, Gadidae) by sequencing and PCR–RFLP analysis of mitochondrial 12S and 16S rRNA gene fragments. *European Food Research and Technology*, **225**, 337-344.

Di Pinto, A., Forte, V.T., Guastadisegni, V.C., Martino, C., Schena, F.P. & Tantillo, G. (2007). A comparison of DNA extraction methods for food analysis. *Food Control*, **18**, 76-80.

DJC (Department of Justice, Canada) (1985a). Food and Drugs Act. R.S., C., F-27. [Internet document]. URL http://laws-lois.justice.gc.ca/en/F-27/C.R.C.-c.870. 12 August 2010.

DJC (Department of Justice, Canada) (1985b). Consumer Packaging and Labelling Act. R.S., C., C-38. [Internet document]. URL http://laws-lois.justice.gc.ca/en/C38/C.-R.C.-c.417. 12 August 2010.

Dobson, R. & Mesure, S. (2007). Anyone for fillet of slimehead? Thought not. *The Independent*, September 23, 2007.

DOH (Department of Health) (2010). Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act 54 of 1972), regulations relating to the labelling and advertising of foodstuffs (R. 146/2010). *South African Government Gazette*, **32975**, 3-53.

Dooley, J.J., Sage, H.D., Clarke, M.L., Brown, H.M. & Garrett, S.D. (2005a). Fish species identification using PCR-RFLP analysis and lab-on-chip capillary electrophoresis: application to detect white fish species in food products and an interlaboratory study. *Journal of Agricultural and Food Chemistry*, **53**, 3348-3357.

Dooley, J.J., Sage, H.D., Brown, H.M. & Garrett, S.D. (2005b). Improved fish species identification by use of lab-on-a-chip technology. *Food Control*, **16**, 601-607.

DTI (Department of Trade and Industry) (2003). Compulsory Specification for frozen fish, frozen marine molluscs and frozen products derived therefrom, VC 8017:2003, Ed. 3 (R. 979/2003). *Government Gazette*, **25172**, 3-50.

DTI (Department of Trade and Industry) (2009). Consumer Protection Act. Act no. 68 of 2008. R. 467/2009. *Government Gazette*, **32186**, 1-186.

Ebach, M.C. & Holdredge, C. (2005). DNA barcoding is no substitute for taxonomy. *Nature*, **434**, 697.

EC (European Commission) (2000a). Directive 2000/13/EC of the European Parliament and of the Council of 20 March 2000 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs. *Official Journal of the European Communities*, **L109**, 29-42.

EC (European Commission) (2000b). European Council Regulation N 104/2000 of 17 December 1999 on the common organization of the markets in fishery and aquaculture products. *Official Journal of the European Communities*, **L17**, 22-52.

EC (European Commission) (2001). Commission Regulation (EC) No 2065/2001 of 22 October 2001 laying down detailed rules for the application of Council Regulation (EC) No 104/2000 as regards informing consumers about fishery and aquaculture products. *Official Journal of the European Communities*, **L278**, 6-8.

EC (European Communities) (2002). Trade description of sardines, 2nd written submission of the European Communities. [Internet document]. URL http://trade.ec.europa.eu/wtodispute/show.cfm?id=242&code=2#_eusubmission. 19 March 2010.

EC (European Communities) (2003a). Labelling of Fishery and Aquaculture Products. Regulation S.I. No. 320 of 2003. [Internet document]. URL www.fsai.ie/uplo-adedFiles/S.I_No_320_of_2003.pdf. 24 July 2010.

EC (European Commission) (2003b). Regulation (EC) No. 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. *Official Journal of the European Communities*, **L268**, 1-23.

Edwards, M.C. & Gibbs, R.A. (1994). Multiplex PCR: advantages, development and applications. *PCR Methods and Applications*, **3**, S65-S75.

Etienne, M., Jérome, M., Fleurence, J., Rehbein, H., Kündiger, R., Mendes, R., Costa, H., Pérez-Martín, R., Piñeiro-González, C., Craig, A., Mackie, I., Yman, M.I., Ferm, M., Martinez, I., Jessen, F., Smelt, A. & Luten, J. (2000). Identification of fish species after cooking by SDS-PAGE and urea IEF: a collaborative study. *Journal of Agricultural and Food Chemistry*, **48**, 2653-2658.

Etienne, M., Jérome M., Fleurence J., Rehbein H., Kündiger R., Mendes R., Costa H. & Martinez, I. (2001). Species identification of formed fishery products and high pressure treated fish by electrophoresis: a collaborative study. *Food Chemistry*, **72**, 105-112.

FAO (Food and Agriculture Organization of the United Nations) (2000). Trends in world fisheries and their resources: 1974-1999. In: *The State of World Fisheries and Aquaculture 2000*. Pp. 98-104. Rome, Italy: FAO.

FAO (Food and Agriculture Organization of the United Nations) (2007). *The State of World Fisheries and Aquaculture 2006*. Pp. 1-160. Rome, Italy: FAO.

FAO (Food and Agriculture Organization of the United Nations) (2008). *FAO Yearbook of Fishery and Aquaculture Statistics 2006*. Pp. 1-57. Rome, Italy: FAO.

FAO (Food and Agriculture Organization of the United Nations) (2009a). *The State of World Fisheries and Aquaculture 2008*. Pp. 1-175. Rome, Italy: FAO.

FAO (Food and Agriculture Organization of the United Nations) (2009b). *FAO Yearbook of Fishery and Aquaculture Statistics 2007*. Pp. 1-72. Rome, Italy: FAO.

FAO (Food and Agriculture Organization of the United Nations) (2010). Fishery and aquaculture country profiles: South Africa national fishery sector overview. [Internet document]. URL www.fao.org/fishery/countrysector/FI-CP_ZA/en. 12 July 2010.

FDA (Food and Drug Administration) (2005). Federal Food, Drug and Cosmetic Act. Section 403 (21 USC §343): Misbranded Food. Washington DC, USA: Government Printing Office.

FDA (Food and Drug Administration) (2009a). FDA's examples of substitute seafood. [Internet document]. URL www.fda.gov/Food/FoodSafety/ProductSpecificInform-ation/Seafood/Regulatory-FishEncyclopediaRFE/ucm071528.htm. 21 May 2010.

FDA (Food and Drug Administration) (2009b). The seafood list - FDA's guide to acceptable market names for seafood sold in interstate commerce. [Internet document]. URL www.fda.gov/Food/GuidanceComplianceRegulatoryInform-ation/GuidanceDocuments/Seafood/ucm113260.htm. 21 May 2010.

Feldman, K.A., Werner, S.B., Cronan, S., Hernandez, M., Horvath, A.R., Lea, C.S., Au, A.M. & Vugia, D.J. (2005). A large outbreak of scombroid fish poisoning associated with eating escolar fish (*Lepidocybium flavobrunneum*). *Epidemiology and Infection*, **133**, 29-33.

Ferguson, A., Taggart, J.B., Prodöhl, P.A., McMeel, O., Thompson, C., Stone, C., McGinnity, P. & Hynes, R.A. (1995). The application of molecular markers to the study and conservation of fish populations, with special reference to *Salmo*. *Journal of Fish Biology*, **47**, S103-S126.

Forrest, A.R.R. & Carnegie, P.R. (1994). Identification of gourmet meat using FINS (Forensically Informative Nucleotide Sequencing). *BioTechniques*, **17**, 24-25.

Foulke, J.E. (1993). Is something fishy going on? Intentional mislabeling of fish. *FDA Consumer*, **27**, 12-17.

Froese, R. & Pauly, D. (2010). FishBase: world wide web electronic publication, version 01/2010. [Internet document]. URL www.fishbase.org. 21 September 2010.

FSANZ (Food Standards Australia New Zealand) (1991). Food Standard Australia New Zealand Act. Act no. 118 of 1991. Pp. 1-149. Canberra, Australia: Office of Legislative Drafting.

FSANZ (Food Standards Australia New Zealand) (2003). A pilot survey on the identity of fish species as sold through food outlets in Australia. [Internet document]. URL www.foodstandards.govt.nz/_srcfiles/FishSpeciationReportfinal.pdf. 20 May 2010.

Fujita, K. & Silver, J. (1994). Single-strand conformational polymorphism. *PCR Methods and Applications*, **4**, S137-S139.

Funk, D.J. & Omland, K.E. (2003). Species-level paraphyly and polyphyly: frequency, causes and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology, Evolution and Systematics*, **34**, 397-423.

Gallardo, J.M., Sotelo, C.G., Piñeiro, C. & Pérez-Martín, R.I. (1995). Use of capillary zone electrophoresis for fish species identification: differentiation of flatfish species. *Journal of Agricultural and Food Chemistry*, **43**, 1238-1244.

Gallic, B.L. & Cox, A. (2006). An economic analysis of illegal, unreported and unregulated (IUU) fishing: key drivers and possible solutions. *Marine Policy*, **30**, 689-695.

Gil, L.A. (2007). PCR-based methods for fish and fishery products authentication. *Trends in Food Science and Technology*, **18**, 558-566.

Givney, R.C. (2002). Illness associated with rudderfish/escolar in South Australia. *Communicable Disease Intelligence*, **26**, 440.

Gray, W.M. (1989). Origin and evolution of mitochondrial DNA. *Annual Review of Cell Biology,* **5**, 25-50.

Gregory, J. (2002). Outbreaks of diarrhoea associated with butterfish in Victoria. *Communicable Disease Intelligence*, **26**, 439-440.

Gulbrandsen, L.H. (2009). The emergence and effectiveness of the Marine Stewardship Council. *Marine Policy*, **33**, 654-660.

Guo, J., Zeng, E., Wu, F., Meng, X., Mai, B. & Luo, X. (2007). Organochlorine pesticides in seafood products from southern China and health risk assessment. *Environmental Toxicology and Chemistry*, **26**, 1109-1115.

Hajibabaei, M., deWaard, J.R., Ivanova, N.V., Ratnasingham, S., Dooh, R.T., Kirk, S.L., Mackie, P.M. & Hebert, P.D.N. (2005). Critical factors for assembling a high volume of DNA barcodes. *Philosophical Transactions of the Royal Society B*, **360**, 1959-1967.

Hall, H.J. & Nawrocki, L.W. (1995). A rapid method for detecting mitochondrial DNA variation in the brown trout, *Salmo trutta*. *Journal of Fish Biology*, **46**, 360-364.

Hannesson, R. (1996). *Fisheries Mismanagement: The Case of the North Atlantic Cod*. Pp. 1-155. Oxford, UK: Fishing News Books.

Hansen, M.M. & Loeschcke, V. (1996). Genetic differentiation among Danish brown trout populations, as detected by RFLP analysis of PCR amplified mitochondrial DNA segments. *Journal of fish Biology*, **48**, 422-436.

Harris, J.D. (2003). Can you bank on GenBank? *Tends in Ecology and Evolution*, **18**, 317-319.

Hayashi, K. (1991). PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. *PCR Methods and Applications*, **1**, 34-38.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & deWaard, J.R. (2003a). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B*, **270**, 313-321.

Hebert, P.D.N, Ratnasingham, S. & deWaard, J.R. (2003b). Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society B*, **270**, S96-S99.

Heemstra, P.C. & Randall, J.E. (1993). FAO species catalogue: groupers of the world. In: *FAO Fisheries Synopsis*, No. 125, Vol. 16. Pp. 1-382. Rome, Italy: FAO.

Herman, L. (2001). Determination of the animal origin of raw food by species-specific PCR. *Journal of Dairy Research*, **68**, 429-436.

Hilborn, R. (2007). Moving to sustainability by learning from successful fisheries. *Ambio*, **36**, 296-303.

Hird, H.J., Hold, G.L., Chrisholm, J., Reece, P., Russell, V.J., Brown, J., Goodier, R. & MacArthur, R. (2005). Development of a method for the quantification of haddock (*Melanogrammus aeglefinus*) in commercial products using real-time PCR. *European Food Research and Technology*, **220**, 633-637.

Hites, R.A., Foran, J.A., Carpenter, D.O., Hamilton, M.C., Knuth, B.A. & Schwager, S.J. (2004). Global assessment of organic contaminants in farmed salmon. *Science*, **303**, 226-229.

Hold, G.L., Russell, V.J., Pryde, S.E., Rehbein, H., Quinteiro, J., Vidal, R., Rey-Méndez, M., Sotelo, C.G., Pérez-Martín, R.I., Santos A.T. & Rosa C. (2001). Development of a DNA-based method aimed at identifying the fish species present in food products. *Journal of Agricultural and Food Chemistry*, **49**, 1175-1179.

Holland, P.M., Abramson, R.D., Watson, R. & Gelfand, D.H. (1991). Detection of specific polymerase chain reaction product by utilizing the 50-30 exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America*, **88**, 7276-7280.

Holzhauser, T., Stephan, O. & Vieths, S. (2006). Polymerase chain reaction (PCR) methods for the detection of allergenic foods. In: *Detecting Allergens in Foods* (edited by S.J. Koppelman & S.L. Hefle). Pp. 125-141. England, UK: Woodhead Publishing Limited.

Hooper, L., Thompson, R.L., Harrison, R.A., Summerbell, C.D., Ness, A.R., Moore, H.J., Worthington, H.V., Durrington, P.N., Higgins, J.P., Capps, N.E., Riemersma, R.A., Ebrahim, S.B. & Davey Smith, G. (2006). Risks and benefits of omega-3 fats for mortality, cardiovascular disease and cancer: a systematic review. *British Medical Journal*, **332**, 752-755.

Hsieh, Y., Woodward, B.B. & Blanco, A.W. (1995). Species substitution of retail snapper fillets. *Journal of Food Quality*, **18**, 131-140.

Hsieh, Y.W., Hwang, P.A., Pan, H.H., Chen, J.B. & Hwang, D.F. (2003). Identification of tetrodotoxin and fish species in an adulterated dried mullet roe implicated in food poisoning. *Journal of Food Science*, **68**, 142-146.

Hsieh, H.S., Chai, T., Cheng, C.A., Hsieh, Y.W. & Hwang, D.F. (2004). Application of DNA technique for identifying the species of different processed products of swordfish meat. *Journal of Food Science*, **69**, 1-6.

Hsieh, H.S., Chai, T.J. & Hwang, D.F. (2005). Rapid PCR-RFLP method for the identification of 5 billfish species. *Journal of Food Science*, **70**, 246-249.

Hsieh, H.S., Chai, T. & Hwang, D.F. (2007). Using the PCR-RFLP method to identify the species of different processed products of billfish meats. *Food Control*, **18**, 369-374.

Hubalkova, Z., Kralik, P., Tremlova, B. & Rencova, E. (2007). Methods of gadoid fish species identification in food and their economic impact in the Czech Republic: a review. *Veterinarni Medicina*, **52**, 273-292.

Hubert, N., Torrico, J.P., Bonhomme, F. & Renno, J.F. (2007). Species polyphyly and mtDNA introgression among three Serrasalmus sister-species. *Molecular Phylogenetics and Evolution*, **46**, 375-381.

Hubert, N., Hanner, R., Holm, E., Mandrak, N.E., Taylor, E., Burridge, M., Watkinson, D., Dumont, P., Curry, A., Bentzen, P., Zhang, J., April, J. & Bernatchez, L. (2008). Identifying Canadian freshwater fishes through DNA barcodes. *PLoS One*, **3**, 2490.

Hutchings, J.A. & Reynolds, J.D. (2004). Marine fish population collapses: consequences for recovery and extinction risk. *Bioscience*, **54**, 297-309.

Iles, A. (2004). Making seafood sustainable: merging consumption and citizenship in the United States. *Science and Public Policy*, **31**,127-138.

IUCN (International Union for Conservation of Nature) (2006). IUCN Red List of Threatened Species. [Internet document]. URL www.iucnredlist.org. 04 May 2010.

Jacquet, J.L. (2009). Fish as food in an age of globalization. PhD Thesis, University of British Columbia, Canada.

Jacquet, J.L. & Pauly, D. (2007). The rise of seafood awareness campaigns in an era of collapsing fisheries. *Marine Policy*, **31**, 308-313.

Jacquet, J.L. & Pauly, D. (2008a). Trade secrets: renaming and mislabelling of seafood. *Marine Policy*, **32**, 309-318.

Jacquet, J.L. & Pauly, D. (2008b). Funding priorities: big barriers to small-scale fisheries. *Conservation and Policy*, **22**, 832-835.

Jacquet, J., Hocevar, J., Lai, S., Majluf, P., Pelletier, N., Pitcher, T., Sala, E., Sumaila, R. & Pauly, D. (2009). Conserving wild fish in a sea of market-based efforts. *Oryx*, **44**, 45-56.

Janzen, D.H. (2004). Now is the time. *Philosophical Transactions of the Royal Society B*, **359**, 731-732.

Jérôme, M., Lemaire, C., Bautista, J.M., Fleurence, J. & Etienne, M. (2003). Molecular phylogeny and species identification of sardines. *Journal of Agricultural and Food Chemistry*, **51**, 43-50.

Jin, L.G., Cho, J.G., Seong, K.B., Park, J.Y., Kong, I.S. & Hong, Y.K. (2006). 18S rRNA gene sequences and random amplified polymorphic DNA used in discriminating

Manchurian trout from other freshwater salmonids. *Fisheries Science*, **72**, 903-905.

Johansen, B., Johnsen, O.C. & Valla, S. (1989). The complete nucleotide sequence of the growth hormone gene from Atlantic salmon (*Salmo salar*). *Gene*, **77**, 317-324.

Kaiser, M.J. & Edward-Jones, G. (2006). The role of ecolabeling in fisheries management and conservation. *Conservation Biology*, **20**, 393-398.

Kinkade, S. & Verclas, K. (2008). *Wireless Technology for Social Change: Trends in Mobile Use by NGOs*. Pp. 48-51. Washington DC, USA: UN Foundation - Vodafone Group Foundation Partnership. [Internet document]. URL http://mobileactive.org/files/MobilizingSocialChange_full.pdf. 12 May 2010.

Kitano, T., Umetsu, K., Tian, W. & Osawa, M. (2007). Two universal primer sets for species identification among vertebrates. *International Journal of Legal Medicine,* **121**, 423-427.

Kleinsmith, L.J. & Kish, V.M. (1995). *Principles of Cell and Molecular Biology*, 2nd ed. Pp.1-810. New York: Harper Collins College Publishers.

Knecht, B. (2006). *Hooked: Pirates, Poaching and the Perfect Fish*. Pp. 1-267. New York, USA: Rodale Books.

Knoth, K., Roberds, S., Poteet, D. & Tamkun, M. (1988). Highly degenerate, inosine-containing primers specifically amplify rare cDNA using the polymerase chain reaction. *Nucleic Acids Research*, **16**, 10932.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S., Paabom S., Villablanca, F.X. & Wilson, A.C. (1989). Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences*, **66**, 6196-6200.

Lack, M. & Sant, G. (2001). Patagonian toothfish: are conservation and trade measures working? *TRAFFIC Bulletin*, **19**, 1-17.

Lam, V. (2007). Oilfish - the case of the imitation Atlantic cod. *Sea Around Us Project Newsletter*, **40**, 1-2.

Laube, I. (2010). Meat. In: *Molecular Biological and Immunological Techniques and Applications for Food Chemists* (edited by B. Popping, C. Diaz-Amigo & K. Hoenicke). Pp. 135-145. New Jersey, USA: John Wiley & Sons, Inc.

Laws, F. (2007). House acts on mislabeled fish imports. *Delta Farm Press*, October 12, 2001. [Internet document]. URL http://deltafarmpress.com/house-acts-mislabeled-fish-imports. 11 September 2010.

LeBlanc, E.L. & LeBlanc, R.J. (1994). Capillary zone electrophoresis of fish muscle sarcoplasmic proteins. *Journal of Food Science*, **59**, 1267-1270.

Lenstra, J.A. (2003). DNA methods for identifying plant and animal species in food. In: *Food Authenticity and Traceability* (edited by M. Lees). Pp. 34-53. Florida, USA: CRC Press.

Lewison, R.L., Freeman, S.A. & Crowder, L.B. (2004). Quantifying the effects of fisheries on threatened species: the impact of pelagic longlines on loggerhead and leatherback sea turtles. *Ecology Letters*, **7**, 221-231.

Lin, L., Cheng, I.P., Tzeng, C.S. & Huang, P.C. (1990). Maternal transmission of mitochondrial DNA in ducks. *Biochemical and Biophysical Research Communications*, **168**, 188-93.

Lin, W.F. & Hwang, D.F. (2008). Application of species-specific PCR for the identification of dried bonito product (Katsuobushi). *Food Chemistry*, **106**, 390-396.

Lockley, A.K. & Bardsley, R.G. (2000a). DNA-based methods for food authentication. *Trends in Food Science and Technology*, **11**, 67-77.

Lockley, A.K. & Bardsley, R.G. (2000b). Novel method for the discrimination of tuna (*Thunnus thynnus*) and bonito (*Sarda sarda*) DNA. *Journal of Agricultural and Food Chemistry*, **48**, 4463-4468.

Logan, C.A., Alter, S.E., Haupt, A.J., Tomalty, K. & Palumbi, S.R. (2008). An impediment to consumer choice: overfished species are sold as Pacific red snapper. *Biological Conservation*, **141**, 1591-1599.

Lopera-Barrero, N.M., Povh, J.A., Ribeiro, R.P., Gomes, P.C., Jacometo, C.B. & Da Silva Lopes, T. (2008). Comparison of DNA extraction protocols of fish fin and larvae samples: modified salt (NaCl) extraction. *Ciencia e InvestigacìÌn Agraria*, **35**, 65-74.

López, I. & Pardo, M.A. (2005). Application of relative quantification Taqman real-time polymerase chain reaction technology for the identification and quantification of *Thunnus alalunga* and *Thunnus albacares*. *Journal of Agricultural and Food Chemistry*, **53**, 4554-4560.

López-Calleja, I., González, I., Fajardo, V., Martín, I., Hernández, P.E., García, T. & Martín, R. (2007). Real-time taqman PCR for quantitative detection of cows' milk in ewes' milk mixtures. *International Dairy Journal,* **17**, 729-736.

Lorenz, J.G., Jackson, W.E., Beck, J.C. & Hanner, R. (2005). The problems and promise of DNA barcodes for species diagnosis of primate biomaterials. *Philosophical Transactions of the Royal Society B*, **360**, 1869-1877.

Love, M.S., Yoklavich, M. & Thorsteinson, L.K. (2002*). Rockfishes of the Northeast Pacific.* Pp. 1-422. California, USA: University of California Press.

Lundstrom, R.C. (1980). Fish species identification by thin-layer polyacrylamide-gel isoelectric-focusing - collaborative study. *Journal of the Association of Official Analytical Chemists*, **63**, 69-73.

Mackie, I.M. (1996). Authenticity of fish. In: *Food Authentication* (edited by P.R. Ashurt & M.J. Dennis). Pp. 140-170. London, UK: Blackie Academic and Professional.

MacLean, C.H., Newberry, S.J., Mojica, W.A. Khanna, P., Issa, A.M., Suttorp, M.J., Lim, M.J., Traina, S.B., Hilton, L., Garland, R. & Morton, S.C. (2006). Effects of omega-3 fatty acids on cancer risk: a systematic review. *Journal of the American Medical Association*, **295**, 403-415.

Marean, C.W., Bar-Matthews, M., Bernatchez, J., Risher, E., Goldberg, P., Herries, A.I.R., Jacobs, Z., Jerardino, A., Karkanas, P., Minichillo, T., Nilssen, P.J., Thompson, E., Wattes, I. & Williams, H.M. (2007). Early human use of marine resources and pigment in South Africa during the Pleistocene. *Nature*, **449**, 905-908.

Marko, P.B., Lee, S.C., Rice, A.M., Gramling, J.M., Fitzhenry, T.M., McAalister, J.S., Harper, G.R. & Moran, A.L. (2004). Mislabelling of a depleted reef fish. *Nature*, **430**, 309-310.

Martinez, I. (2003). Application of protein electrophoretic analyses and DNA fingerprinting to species identification. In: *Authenticity of Species in Meat and Seafood Products* (edited by R. Pérez-Martín & C.G. Sotelo)*.* Pp. 177-188. Vigo, Spain: International Congress on Authenticity of Species in Meat and Seafood Products Association.

Martinez, I., Christiansen, J.S., Ofstad, R. & Olsen, R.L. (1991). Comparison of myosin isoenzymes present in skeletal and cardiac muscles of the Arctic charr *Salvelinus alpinus* L: sequential expression of different myosin heavy chains during development of the fast white skeletal muscle. *European Journal of Biochemistry*, **195**, 743-753.

Martinez, I. & Yman, M.I. (1998). Species identification in meat products by RAPD analysis. *Food Research International*, **31**, 459-466.

Martinez, I., Friis, T.J. & Seppola, M. (2001). Requirements for the application of protein SDS-PAGE and RAPD to product speciation. *Electrophoresis*. **22**, 1526-1533.

Martinez, I., James, D. & Loréal, H. (2005). Application of modern analytical techniques to ensure seafood safety and authenticity. *FAO Fisheries Technical Paper No. 455*. Pp. 1-73. Rome, Italy: FAO.

Martinez-Ortiz, J. (2005). *Whitefish Handbook of Ecuador: 45 Species of Commercial Interest.* Quito, Ecuador: Asoexpleba (As cited by Jacquet & Pauly, 2007).

McDowell, J. & Graves, J.E. (2002). Nuclear and mitochondrial DNA markers for specific identification of istiophorid and xiphiid billfishes. *Fishery Bulletin*, **100**, 537-544.

McMichael, A.J. & Butler, C.D. (2005). Fish, health, and sustainability. *American Journal of Preventive Medicine*, **29**, 322-323.

Merente, F., Raha, S. & Ling, M. (1998). Isolation of total cellular DNA from tissue and cultured cells. In: *Molecular Biomethods Handbook* (edited by R. Rapley & J.M. Walker). Pp. 9-16. New Jersey, USA: Humana Press Inc.

Meyer, R., Höfelein, C., Lüthy, J. & Candrian, U. (1995). Polymerase chain reaction-restriction fragment length polymorphism analysis: a simple method for species identification in food. *Journal of the AOAC International*, **78**, 1542-1551.

Miller, B. (1981). Fish with an image problem. *New York Times*, September 23, 1981.

Miya, M., Takeshima, H., Endo, H., Ishiguro, N.B., Inoue, J.G., Mukai, T., Satoh, T.P., Yamaguchi, M., Kawaguchi, A., Mabuchi, K., Shirai, S.M. & Nishida, M. (2003). Major patterns of higher teleostean phylogenies: a new perspective based on 100 complete mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, **26**, 121-138.

Mok, D. (2007). Welcome sold sushi and sashimi made from oilfish. *The South China Morning Post*, February 2, 2007.

Montoro, A., Perteguer, M.J., Chivato, T., Laguna, R. & Cuellar, C. (1997). Recidivous acute urticaria caused by *Anisakis simplex*. *Allergy*, **52**, 985-991.

Moreno-Ancillo, A., Caballero, M.T., Cabañas, R., Contreras, J., Martin-Barroso, J.A., Barranco, P. & López-Serrano, M.C. (1997). Allergic reactions to *Anasakis simplex* parasitizing seafood. *Annals of Allergy, Asthma and Immunology*, **79**, 246-50.

Moretti, V.M., Turchini, G.M., Bellagamba, F. & Caprino, F. (2003). Traceability issues in fishery and aquaculture products. *Veterinary Research Communciations*, **27**, 497-505.

Mori, M., Saito, T., Nakanishi, Y., Miyazawa, K. & Hashimoto, Y. (1966). The composition and toxicity of wax in the flesh of castor oil fishes. *Bulletin of the Japanese Society of Scientific Fisheries*, **32**, 137-145.

Moritz, C. & Cicero, C. (2004). DNA barcoding: promise and pitfalls. *PLoS Biology*, **2**, 1529-1531.

Mullis, K.B., Faloona, F.A., Scharf, S.J., Saiki, R.K., Horn, G.T. & Erlich, H.A. (1986). Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harbor Symposium on Quantitative Biology,* **51**, 263-273.

Murgia, R., Tola, G., Archer, S.N., Vallerga, S. & Hirano, J. (2002). Genetic identification of grey mullet species (*Mugilidae*) by analysis of mitochondrial DNA sequence: application to identify the origin of processed ovary products (Bottarga). *Marine Biotechnology*, **4**, 119-126.

Myers, R.A. & Worm, B. (2003). Rapid worldwide depletion of predatory fish communities. *Nature*, **423**, 280-283.

Naylor, R.L., Goldburg, R.J., Mooney, H., Beveridge, M., Clay, J., Folke, C., Kautsky, N., Lubchenco, J., Primavera, J. & Williams, M. (1998). Nature's subsidies to shrimp and salmon farming. *Nature*, **282**, 883-884.

Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J., Folke, C., Lubchenco, J., Mooney, H. & Troell, M. (2000). Effect of aquaculture on world fish supplies. *Nature*, **405**, 1017-1024.

Necidová, L., Rencová, E. & Svoboda, I. (2002). Counter immuno-electrophoresis: a simple method for the detection of species-specific muscle proteins in heat-processed products. *Veterinární Medicína*, **47**, 143-147.

NET (National Environmental Trust) (2004). Black market for white gold: the illegal trade in Chilean sea bass. [Internet document]. URL www.illegal-fishing.info/-uploads/Black-mkt-for-white-gold-csb-report.pdf. 10 November 2010.

Nohlgren S. (2006). How to prove it's grouper? *St. Petersbug Times*, December 6, 2006.

Nohlgren, S. & Tomalin, T. (2006). You order grouper; what do you get? *St. Petersburg Times*, August 6, 2006.

Nohlgren, S. & Tomalin, T. (2007). Red grouper stocks rebound - but wait. *St. Petersburg Times*, February 7, 2007.

Oohara, I. (1997). Detection of single strand conformation polymorphisms (SSCPs) on mitochondrial DNA fragments between two domesticated strains of rainbow trout *Oncorhynchus mykiss*. *Fisheries Science*, **63**, 151-152.

Orita, M., Suzuki, Y., Sekiya, T. & Hayashi, K. (1989). A rapid and sensitive detection of point mutations and genetic polymorphisms using polymerase chain reaction. *Genomics*, **5**, 874-879.

Osman, M.A., Ashoor S.H. & Marsh, P.C. (1987). Liquid chromatographic identification of common fish species. *Journal of the Association of Official Analytical Chemists*, **70**, 618-625.

Pardo, M.A. & Pérez-Villareal, B. (2004). Identification of commercial canned tuna species by restriction site analysis of mitochondrial DNA products obtained by nested primer PCR. *Food Chemistry*, **86**, 143-150.

Partis, L. & Wells, R.J. (1996). Identification of fish species using random amplified polymorphic DNA (RAPD). *Molecular and Cellular Probes*, **10**, 435-441.

Pauly, D., Christensen, V., Dalsgaard, J., Froese, R. & Torres, F. (1998). Fishing down marine food webs. *Science*, **279**, 860-863.

Pauly, D., Alder, J., Bennett, E., Christensen, V., Tyedmers, P. & Watson, R. (2003). The future for fisheries. *Science*, **302**, 1359-1361.

Pauly, D., Watson, R. & Alder, J. (2005). Global trends in world fisheries: impacts on marine ecosystems and food security. *Philosophical Transactions of the Royal Society B*, **360**, 5-12.

Phillips, B., Ward, T. & Chaffee, C. (2003). *Eco-labelling in Fisheries: What is it All About?* Pp. 1-196. Oxford, UK: Blackwell Publishing.

Piñeiro, C., Barros-Velázquez, J., Sotelo, C.G., Pérez-Martín, R.I. & Gallardo, J.M. (1998). Two dimensional electrophoretic study of the water-soluble protein fraction in white muscle of Gadoid fish species. *Journal of Agricultural and Food Chemistry*, **46**, 3991-3997.

Piñeiro, C., Barros-Velázquez, J., Pérez-Martín, R.I., Martinez, I., Jacobsen, T., Rehbein, H., Kündiger, R., Mendes, R., Etienne, M., Jerome, M., Craig, A., Mackie, I.M., & Jessen, F. (1999). Development of a SDS-PAGE reference method for the analysis and identification of fish species in raw and heat-processed samples: a collaborative study. *Electrophoresis*, **20**, 1425-1432.

Pitcher, T. & Pauly, D. (1998). Rebuilding ecosystems, not sustainability, as the proper goal of fishery management. In: *Reinventing Fisheries Management* (edited by T. Pitcher, P. Hart & D. Pauly). Pp. 311-329. Dordrecht, The Netherlands: Kluwer Academic Publishers.

Pitcher, T.J., Watson, R., Forest, R., Valtysson, H. & Guenette, S. (2002). Estimating illegal and unreported catches from marine ecosystems: a basis for change. *Fish and Fisheries*, **3**, 317-339.

Pleijel, F., Jondelius, U., Norlinder, E., Nygren, A., Oxelman, B., Schander, C., Sundberg, P. & Thollesson, M. (2008). Phylogenies without roots? A plea for the use of vouchers in molecular phylogenetic studies. *Molecular Phylogenetics and Evolution*, **48**, 369-371.

Ponte, S. (2008). Greener than thou: the political economy of fish ecolabeling and its local manifestations in South Africa. *World Development*, **36**, 159-175.

Puyet, A. & Bautista, J.M. (2010). Detection of adulterations: identification of seafood species. In: *Handbook of Seafood and Seafood Product Analysis* (edited by L.M.L. Nollet & F. Toldra). Pp. 688-704. Florida, USA: CRC Press.

Quinteiro, J., Sotelo, C.G., Rehbein, H., Pryde, S.E., Medina, I., Pérez-Martín, R.I., Rey-Méndez, M. & Mackie, I.M. (1998). Use of mtDNA direct polymerase chain reaction (PCR) sequencing and PCR-restriction fragment polymorphism methodologies in species identification of canned tuna. *Journal of Agricultural and Food Chemistry*, **46**, 1662-1669.

Quinteiro, J., Vidal, R., Izquierdo, V., Sotelo, C.G., Chapela, M.J., Pérez-Martín, R.I., Rehbein, H., Hold, G.L., Russell, V.J., Pryde, S.E., Rosa, P., Santos, A.T. & Rey-Méndez, M. (2001). Identification of hake species *Merluccius* genus using sequencing and PCR-RFLP analysis of mitochondrial DNA control region sequences. *Journal of Agricultural and Food Chemistry*, **49**, 5108-5114.

Ram, J.L., Ram, M.L. & Baidoun, F.F. (1996). Authentification of canned tuna and bonito sequence and restriction site analysis of polymerase chain reaction products of mitochondrial DNA. *Journal of Agricultural and Food Chemistry*, **44**, 2460-2467.

Randolph, S. & Snyder, M. (1993). The Seafood List: FDA's Guide to Acceptable Market Names for Seafood Sold in Interstate Commerce. Pp. 1-66. Washington DC, USA: Government Printing Office.

Rapley, R. (1998). Polymerase chain reaction. In: *Molecular Biomethods Handbook* (edited by R. Rapley & J.M. Walker). Pp. 305-326. New Jersey, USA: Humana Press Inc.

Rapley, R. (2003). Molecular biology and basic techniques. In: *Practical Biochemistry: Principles and Techniques*, 5th ed. (edited by K. Wilson & J. Walker). Pp. 80-136. Cambridge, UK: Cambridge University Press.

Rasmussen, R.S. & Morrisey, M.T. (2009). Application of DNA-based methods to identify fish and seafood substitution on the commercial market. *Comprehensive Reviews in Food Science and Food Safety*, **8**, 118-154.

Rastogi, G., Dharne, M.S., Walujkar, S., Kumar, A., Patole, M.S. & Shouche, Y.S. (2007). Species identification and authentication of tissues of animal origin using mitochondrial and nuclear markers. *Meat Science*, **76**, 666–674.

Ratnasingham, S. & Hebert, P.D.N. (2007). BOLD: the Barcode of Life Data System. *Molecular Ecology Notes*, **7**, 355-364.

Reed, M. (2006). Florida restaurants fight off fake grouper. *USA Today*, November 21, 2006.

Rehbein, H. (2003). Identification of fish species by protein and DNA-analysis. In: *Authenticity of Species in Meat and Seafood Products* (edited by R.I. Pérez-Martín & C.G. Sotelo). Pp. 83-101. Vigo, Spain: International Congress on Authenticity of Species in Meat and Seafood Products Association.

Rehbein, H. (2005). Fish species identification by peptide mapping of the myosin heavy chain. *Electrophoresis*, **13**, 805-806.

Rehbein, H. (2009). DNA-based methods. In: *Fishery Products: Quality, Safety and Authenticity* (edited by H. Rehbein & J. Oehlenschläger). Pp. 363-380. Oxford, UK: Blackwell Publishing.

Rehbein, H., Etienne, M., Jerome, M., Hattula, T., Knudsen, L.B., Jessen, F., Luten, J.B., Bouquet, W., Mackie, I.M., Ritchie, A.H., Martin, R. & Mendes, R. (1995). Influence of variation in methodology on the reliability of the isoelectric focusing method of fish species identification. *Food Chemistry*, **52**, 193-197.

Rehbein, H., Kress, G. & Schmidt, T. (1997). Application of PCR-SSCP to species identification of fishery products. *Journal of Science of Food and Agriculture*, **74**, 35-41.

Rehbein, H., Mackie, I.M., Pryde, S., Gonzales-Sotelo, C., Medina, I., Pérez, R., Quinteiro, J. & Rey-Méndez, M. (1999). Fish species identification in canned tuna by PCR-SSCP: validation by a collaborative study and investigation of intra-species variability of the DNA-patterns. *Food Chemistry*, **64**, 263-268.

Rehbein, H. & Horstkotte, B. (2003). Determination of the composition of multi-species fishery products by PCR-based techniques. In: *Proceedings of the First Trans-Atlantic Fisheries Technology Conference*. Pp. 190-192. June 2003. Reykjavik, Iceland.

Rehbein, H. & Oehlenschläger, J. (2009). Basic facts and figures. In: *Fishery Products: Quality, Safety and Authenticity* (edited by H. Rehbein & J. Oehlenschläger). Pp. 1-15. Oxford, UK: Blackwell Publishing.

Rex, E. & Baumann, H. (2007). Beyond ecolabels: what green marketing can learn from conventional marketing. *Journal of Cleaner Production*, **15**, 567-576.

Rezzi, S., Giani, I., Héberger, K., Axelson, D.E., Moretti, V.M., Reniero, F. & Guillou, C. (2007). Classification of gilthead sea bream (*Sparus aurata*) from ^{1}H NMR lipid profiling combined with principal component and linear discriminant. *Journal of Agricultural and Food Chemistry*, **55**, 9963-9968.

Richardson, D.E., Vanwye, J.D., Exum, A.M., Cowen, R.K. & Crawford, D.L. (2007). High-throughput species identification: from DNA isolation to bioinformatics. *Molecular Ecology Notes*, **7**, 199-207.

Rock, J., Costa, F.O., Walker, D.I., North, A.W., Hutchinson, W.F. & Carvalho, G.R. (2008). DNA barcodes of fish of the Scotia Sea, Antarctica indicate priority groups for taxonomic and systematics focus. *Antarctic Science*, **20**, 253-262.

Roe, S. & Hawthorne, M. (2005). How safe is tuna? *Chicago Tribune*, December 13, 2005.

Roheim, C.A. (2003). Early indications of market impacts from the Marine Stewardship Council's ecolabeling of seafood. *Marine Resource Economics*, **18**, 95-104.

Roheim, C.A. & Sutinen, J. (2006). Trade and marketplace measures to promote sustainable fishing practices. In: *International Trade and Sustainable Development Series Issue Paper No. 3*. Pp. 1-47. Geneva, Switzerland: International Centre for Trade and Sustainable Development and the High Seas Task Force.

Rokas, A., Ladoukakis, E. & Zouros, E. (2003). Animal mitochondrial DNA recombination revisited. *Trends in Ecology and Evolution*, **18**, 411-417.

Roper, M.G., Easley, C.J. & Landers, J.P. (2005) Advances in polymerase chain reaction on microfluidic chips. *Analytical Chemistry*, **77**, 3887-3893.

Rubec, P.J. (1988). The need for conservation and management of Philippine coral reefs. *Environmental Biology of Fishes*, **23**, 141-154.

Rubinoff, D., Cameron, S. & Will, K. (2006). A genomic perspective on the shortcomings of mitochondrial DNA for ''barcoding'' identification. *Journal of Heredity*, **97**, 581-594.

Ruedas, L.A., Salazar-Bravo, J., Dragoo, J.W. & Yates, T.L. (2000). The importance of being earnest: what, if anything, constitutes a "specimen examined?" *Molecular Phylogenetics and Evolution*, **17**, 129-132.

Rychlik, W., Spencer, W.J. & Rhoads, R.E. (1990). Optimization of the annealing temperature for DNA amplification *in vitro*. *Nucleic Acids Research*, **18**, 6409-6412.

Saavedra-Delgado, A.M. & Metcalfe, D.D. (1993). Seafood toxins. *Clinical Reviews in Allergy and Immunology*, **11**, 241-260.

Sackesen, C. & Adalioglu, G. (2003). Hidden fish substance triggers allergy. *Journal of Investigational Allergology and Clinical Immunology*, **13**, 216-217.

Saiki, R.B., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. & Erlich, H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, **239**, 487-491.

Sampson, H.A. (2004). Update on food allergy. *Journal of Allergy and Clinical Immunology*, **113**, 805-819.

SASSI (South African Sustainable Seafood Initiative) (2010). [Internet document]. URL www.wwfsassi.co.za. 14 July 2010.

Sastri, D.C., Hilu, K., Appels, R., Lagudah, E.S., Playford, J. & Baum, B.R. (1992). An overview of evolution in plant 5S DNA. *Plant Systematics and Evolution*, **183**, 169-181.

Saunders, G.C. (1999). DNA extraction. In: *Analytical Molecular Biology: Quality and Validation* (edited by G.C. Saunders & H.C. Parkes). Pp. 29-40. Cambridge, UK: Royal Society of Chemistry.

Savolainen, V., Cowan, R.S., Vogler, A.P., Roderick, G.K. & Lan, R. (2005). Towards writing the encyclopedia of life: an introduction to DNA barcoding. *Philosophical Transactions of the Royal Society B*, **360**, 1805-1811.

Schander, C. & Willassen, E. (2005). What can biological barcoding do for marine biology? *Marine Biology Research*, **1**, 79-83.

Scoggan, K.A. & Bulman, D.E. (2003). Single-strand conformational polymorphism analysis (SSCP) and sequencing for ion channel mutations. In: *Neurogenetics: Methods and Protocols*, Vol. 217 (edited by T. Potter). Pp. 143-150. New Jersey, USA: Humana Press Inc.

SeaWeb (2002). Give swordfish a break! [Internet document]. URL www.seaweb.org/initiatives/swordfish/index.html. 12 March 2010.

Sebastio, P., Zanelli, P. & Neri-Tauro, M. (2001). Identification of anchovy (*Engraulis encrasicholus* L.) and gilt sardine (*Sardinella aurita*) by polymerase chain reaction, sequence of their mitochondrial cytochrome *b* gene, and restriction analysis of polymerase chain reaction products in semipreserves. *Journal of Agricultural and Food Chemistry*, **49**, 1194-1199.

Seberg, O., Humphries, C.J., Knapp, S., Stevenson, D.W., Petersen, G., Scharff, N. & Andersen, N.M. (2003). Shortcuts in systematics? A commentary on DNA-based taxonomy. *Trends in Ecology and Evolution*, **18**, 63-65.

Sevilla, R., Diez, A., Noren M., Mouchel, O., Jérôme, M., Verrez-Bagnis, V., Van Pelt, H., Favre-Krey, L., Krey, G. & Bautista, J.M. (2007). Primers and polymerase chain reaction conditions for DNA barcoding teleost fish based on the mitochondrial cytochrome *b* and nuclear rhodopsin genes. *Molecular Ecology Notes*, **7**, 730-734.

Shadbolt, C., Kirk, M. & Roche, P. (2002). Editorial: Diarrhoea associated with consumption of escolar (rudderfish). *Communicable Disease Intelligence*, **26**, 436-438.

Sotelo, C.G., Piñeiro, C., Gallardo, J.M. & Pérez-Martín, R.I. (1993). Fish species identification in seafood products - review. *Trends in Food Science and Technology*, **4**, 395-401.

Sotelo, C.G., Chapela, M.J., Rey, M. & Pérez-Martín, R. (2003). Development of an identification and quantitation system for cod (*Gadus morhua)* using Taqman assay. In: *Proceedings of the First Trans-Atlantic Fisheries Technology Conference*. Pp.195-198. June, 2003. Reykjavik, Iceland.

Southgate, D.A.T. (2000). Fish and fish products. In: *Human Nutrition and Dietetics* (edited by J.S. Garrow, A. Ralph & W.P.T James). Pp. 369-371. London, UK: Churchill Livingstone.

Spies, I.B., Gaichas, S., Stevenson, D.E., Orr, J.W. & Canino, M.F. (2006). DNA-based identification of Alaska skates (*Amblyraja*, *Bathyraja* and *Raja*: Rajidae) using cytochrome *c* oxidase subunit I (COI) variation. *Journal of Fish Biology*, **69**, S283-S292.

Standal, I.B., Axelson, D.E. & Aursand, M. (2010). ^{13}C NMR as a tool for authentication of different gadoid fish species with emphasis on phospholipid profiles. *Food Chemistry*, **121**, 608-615.

Steinke, D., Zemlak, T.S., Hebert, P.D.N. & Adamowicz, S.A. (2009). DNA barcoding of Pacific Canada's fishes. *Marine Biology*, **156**, 2641-2647.

Stoeckle, M. (2003). Taxonomy, DNA, and the barcode of life. *BioScience*, **53**, 796-797.

Suvanich, V., Marshall, D.L. & Jahncke, M.L. (2000). Microbiological and color quality changes of channel catfish frame mince during chilled and frozen storage. *Journal of Food Science*, **65**, 151-154.

Tautz, D., Arctander, P., Minelli, A., Thomas, R.H. & Vogler, A.P. (2002). DNA points the way ahead in taxonomy. *Nature*, **418**, 479.

Tautz, D., Arctander, P., Minelli, A., Thomas, R.H. & Vogler, A.P. (2003). A plea for DNA taxonomy. *Trends in Ecology and Evolution*, **18**, 70-74.

Taylor, W.J. & Leighton Jones, J. (1992). An immunoassay for verifying the identity of canned sardines. *Food and Agricultural Immunology*, **4**, 169-175.

Teis, M.F., Roe, B. & Levy, A.S. (1999). Eco-certification: why it may not be a ‘‘field of dreams’’. *American Journal of Agricultural Economics*, **81**, 1066-1071.

Teletchea, F. (2009). Molecular identification methods of fish species: reassessment and possible applications. *Reviews of Fish Biology and Fisheries*, **19**, 265-293.

Tennyson, J.M., Winters, K.S. & Powell, K. (1997). A fish by any other name: a report on species substitution. In: *Papers presented at the 22nd Annual Meeting of the Seafood Science and Technology Society of the Americas*. October, 1997. Biloxi, Mississippi.

Tidwell, J.H. & Allan, G.L. (2001). Fish as food: aquaculture’s contribution. *EMBO reports*, **21**, 958-962.

Trotta, M., Schonhuth, S., Pepe, T., Cortesi, M.L., Puyet, A. & Bautista, J.M. (2005). Multiplex PCR method for use in Real-Time PCR for identification of fish fillets from grouper (*Epinephelus* and *Mycteroperca* species) and common substitute species. *Journal of Agricultural and Food Chemistry*, **53**, 2039-2045.

Tserpes, G., Tatamanidis, G. & Peristeraki, P. (2006). Oilfish and shark by-catches of the Greek swordfish fishery in the E. Mediterranean: a preliminary analysis applied to “presence-absence” data. *ICCAT Collective Volume of Scientific Papers*, **59**, 987-991.

Unseld, M., Beyermann, B., Brandt, P. & Hiesel, R. (1995). Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. *PCR Methods and Applications*, **4**, 241-243.

USDA (United States Department of Agriculture) (2009). Mandatory country of origin labeling of beef, pork, lamb, chicken, goat meat, wild and farm-raised fish and shellfish, perishable agricultural commodities, peanuts, pecans, ginseng, and macadamia nuts. Docket No. AMS-LS-07-0081. *Federal Register*, **74**, 2658-2706.

Valdimarsson, G. (2007). Challenges for the global seafood industry. In: *FAO Fisheries Proceedings: Sixth World Congress on Seafood Safety, Quality and Trade.* Pp 17-26. September 2005. Sydney, Australia.

Valenzuela, M.A., Gamarra, N., Gómez, L., Kettlun, A.M., Sardón, M., Pérez, L.M., Vinagre, J. & Guzman, N.A. (1999). A comparative study of fish species identification by gel isoelectrofocusing two-dimensional gel electrophoresis, and capillary zone electrophoresis. *Journal of Capillary Electrophoresis and Microchip Technology*, **6**, 85-91.

Van der Elst, R. (1997). *A Guide to Common Sea Fishes of Southern Africa*. Pp. 7-22. Cape Town, South Africa: Struik Publishers.

Vawter, L. & Brown, W.M. (1986). Nuclear and mitochondrial DNA comparisons reveal extreme rate variation in the molecular clock. *Science*, **234**, 194-196.

Von der Heyden, S., Barendse, J., Seebregts, A.J. & Matthee, C.A. (2010). Misleading the masses: detection of mislabelled and substituted frozen fish products in South Africa. *ICES Journal of Marine Science*, **67**, 176-185.

Walker, J.M. (2003). Electrophoretic techniques. In: *Practical Biochemistry: Principles and Techniques*, 5th ed. (edited by K. Wilson & J. Walker). Pp. 580-618. Cambridge, UK: Cambridge University Press.

Walsh, R. (2001). Fish fraud. *Houston Press*, November 1, 2001. [Internet document]. URL www.houstonpress.com/2001-11-01/restaurants/fish-fraud/. 01 October 2010.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R. & Hebert, P.D.N. (2005). DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B,* **360**, 1847 1857.

Ward, R.D. & Holmes, B.H. (2007). An analysis of nucleotide and amino acid variability in the barcode region of cytochrome *c* oxidase I (*c*ox1) in fishes. *Molecular Ecology Notes*, **7**, 899-907.

Ward, R.D., Holmes, B.H., White, W.T. & Last, P.R. (2008). DNA barcoding Australian chondrichthyans: results and potential uses in conservation. *Marine and Freshwater Research*, **59**, 57-71.

Ward, R.D., Hanner, R. & Herbert, P.D.N. (2009). The campaign to DNA barcode all fishes, FISH-BOL. *Journal of Fish Biology*, **74**, 329-356.

Wasko, A.P., Martins, C., Oliveira, C. & Foresti, F. (2003). Non-destructive genetic sampling in fish. An improved method for DNA extraction from fish fins and scales. *Hereditas*, **138**, 161-165.

Watabe, S., Hirayama, Y., Imai, J., Kikuchi, K. & Yamashita, M. (1995). Sequences of cDNA clones encoding alpha-actin of carp and goldfish skeletal muscles. *Fisheries Science*, **61**, 998-1003.

Weder, J.K., Rehbein, H. & Kaiser, K.P. (2004). On the specificity of tuna-directed primers in PCR-SSCP analysis of fish and meat. *European Food Research and Technology*, **213**, 139-144.

Welsh, J. & McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research*, **18**, 7213-7218.

Wessells, C.R., Johnston, R.J. & Donath, H. (1999). Assessing consumer preferences for ecolabeled seafood: the influence of species, certifier, and household attributes. *American Journal of Agricultural Economics*, **81**, 1084-1089.

WHO (World Health Organization) (2003). Diet, nutrition and the prevention of chronic diseases. In: *WHO Technical Report Series 916*. Pp. 22-23. Geneva, Switzerland: World Health Organization.

Will, K. & Rubinoff, D. (2004). Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. *Cladistics*, **20**, 47-55.

Williams, M. (1996). The transition in the contribution of living aquatic resources to food security. In: *IFPRI 2020 Vision for Food, Agriculture, and the Environment Discussion Paper*, No. 13, Series 32. Pp. 3-24. Washington DC, USA: International Food Policy Research Institute.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J. & Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, **18**, 6531-6535.

Woolfe, M. & Primrose, S. (2004). Food forensics: using DNA technology to combat misdescription and fraud. *Trends in Biotechnology*, **22**, 222-226.

Worm, B., Barbier, E.B. & Beaumont, N. (2006). Impacts of biodiversity loss on ocean ecosystem services. *Science*, **314**, 787-790.

Yakob, M.T., Viljoen, M.F., Jooste, A. & Graz, M. (2006). International trade performance of the South African fish industry. In: *Proceedings of the 2006 Annual meeting of the International Association of Agricultural Economists*. August 2006. Queensland, Australia.

Yamashita, Y., Omura, Y. & Okazaki, E. (2006). Distinct regional profiles of trace element content in muscle of Japanese eel *Anguilla japonica* from Japan, Taiwan, and China. *Fisheries Science*, **72**, 1109-1113.

Yamazaki, Y., Shimada, N. & Tago, Y. (2005). Detection of hybrids between masu salmon *Oncorhynchus masou masou* and amago salmon *O. m. ishikawae* occurred in the Jinzu River using a random amplified polymorphic DNA technique. *Fisheries Science*, **71**, 320- 326.

Yancy, H.F., Zemlak, T.S., Mason, J.A., Washington, J.D., Tenge, B.J., Nguyen, N.L.N.T., Barnett, J.D., Savary, W.E., Hill, W.E., Moore, M.M., Fry, F.S., Randolph, S.C., Rogers, P.L. & Hebert, P.D.N. (2007). The potential use of DNA barcodes in regulatory science: applications of the Regulatory Fish Encyclopedia. *Journal of Food Protection*, **70**, 210-217.

Yue, G.H. & Orban, L. (2001). Rapid isolation of DNA from fresh and preserved fish scales for polymerase chain reaction. *Marine Biotechnology*, **3**, 199-204.

Zardoya, R., Garrido-Pertierra, A. & Bautista, J.M. (1995). The complete nucleotide sequence of the mitochondrial DNA genome of the rainbow trout, *Oncorhynchus mykiss*. *Journal of Molecular Evolution*, **41**, 942-951.

Zeviani, M., Tiramti, V. & Piantadosi, C. (1998). Mitochondrial disorders. *Medicine*, **77**, 59-72.

Zhang, D.X. & Hewitt, G.M. (1996). Nuclear integrations: challenge for mitochondrial DNA markers. *Trends in Ecology and Evolution*, **11**, 247-251.

Zhang, C., Xu, J., Ma, W. & Zheng, W. (2006). PCR microfluidic devices for DNA amplification. *Biotechnology Advances*, **24**, 243-284.

Zhang, C. & Zing, D. (2007). Miniaturized PCR chips for nucleic acid amplification and analysis: latest advances and future trends. *Nucleic Acids Research*, **35**, 4223-4237.

Zinn, C., Lopata, A., Visser, M. & Potter, P.C. (1997). The spectrum of allergy to South African bony fish (Teleosti). *South African Medical Journal*, **87**, 146-151.

CHAPTER 3

EVALUATING THE AVAILABILITY OF FISH SPECIES ON THE SOUTH AFRICAN MARKET AND THE FACTORS UNDERMINING SUSTAINABILITY AND CONSUMER CHOICE

Abstract

Information pertaining to the commercial availability of fish species in South Africa remains sparse, even though it is crucial for consumers to make informed purchasing decisions in favour of their own wellbeing and the wellbeing of the environment. The aim of this study was to determine the most commonly available fish species in South Africa by means of surveys of restaurants (n = 215) and retail outlets (n = 200) and to assess the conservation status of each of the observed species. Furthermore, the processing states in which fish were mostly sold (fresh, frozen, whole or filleted) and the quality of information available to consumers on fish at the point of sale were evaluated. Kingklip was found to be the most commonly marketed fish species in restaurants, while hake was observed most frequently in retail outlets. More than 30% of the observed species were of conservation concern and included, amongst others, kingklip, kabeljou (kob), east coast sole, west coast sole and geelbek. Specially protected, illegal-to-sell fish species in South Africa, such as white steenbras, white musselcracker and Natal stumpnose, were marketed in restaurants and retail outlets. This study highlighted the poor ability of fish purveyors in South Africa to provide information on the identity, origin, production method and sustainability of the fish species being sold. Additionally, the labelling of many packaged fish products in retail outlets was in contravention with South African regulations. Poor vendor awareness, disparate naming practices and the highly processed nature of fishery products provide an opportunity for unintentional or deliberate mislabelling of fish in South Africa. Greater partnership between the fishing industry, fish suppliers, government, environmental organisations and the academia is required to promote sustainability, transparency and fair trade in the local fisheries market.

Cawthorn, D.M., Steinman, H.A. & Witthuhn, R.C. (2011). Evaluating the availability of fish species on the South African market and the factors undermining sustainability and consumer choice. *Food Control*, **22**(11), 1748-1759.

Introduction

South Africa is one of the most important fishing nations in Africa in terms of both fish production and trade (INFOSA, 2007). The total marine fish capture in this country between 2005 and 2008 averaged 689 681 tons (live weight) per annum, which was considerably higher than that derived in the same time period from other prominent fisheries role players on the continent, such as Namibia (*ca*. 466 930 tons per annum) and Angola (*ca*. 264 440 tons per annum). Approximately 21% of the South African catch was exported in 2007 (FAO, 2008). There are four coastal fishing provinces in South Africa, which include Kwazulu-Natal (KZN), the Western Cape (WC) and the Eastern Cape (EC) where most fishing activity is centered, as well as the Northern Cape (NC) where only about 1% of the South African total allowable catch (TAC) is landed (Anon., 2004). Although data on total and species-specific annual catches in South Africa are readily accessible, no studies have been published to date assessing the availability of different fish species on the local market and the quality of information provided on fish products at the point of sale to assist consumer purchasing decisions.

Growing consumer trends towards health have led to a remarkable increase in the consumption of fish and fish products. Coupled with these trends, however, is the desire of consumers to be better informed on the foods they purchase to protect their own wellbeing and that of the environment (Gil, 2007; Grunert, 2002). The many documented health benefits of fish can be offset by the accumulation of toxic contaminants in fish tissue, which may be absorbed from the surrounding marine environment (Costa, 2007). Among the most relevant fish contaminants are methylmercury and organohalogenated compounds. Exposure to these compounds by humans is predominantly associated with reproductive and developmental neurotoxicity (Harada, 2005; NRC, 2000). Since the levels of contaminants in fish are cumulative and increase at each level of the food chain, older predatory fish generally have the highest contaminant concentrations (Chen & Chen, 2001). Consumer health advisories issued by the United States Food and Drug Administration (FDA) advocate that young children, women of childbearing age and pregnant or nursing women should avoid eating four types of fish recognised to be high in mercury (king mackerel, shark, swordfish and tilefish) (FDA, 2004). It has been suggested that the concentration of polychlorinated biphenyls (PCBs) may be higher in farmed salmon than in wild-caught types, since the former are reared on fish meal which could be high in contaminants (Hites *et al*., 2004). In order to make decisions in favour of health, consumers may thus benefit from having comprehensive information available on the species, origin and production method (farmed or wild) of the fish they are purchasing (Burger *et al*., 2004).

Another important issue that might potentially influence consumer selection of certain species is the increasing concern relating to the sustainability of global marine fish resources. Widespread overfishing has led to the collapse of numerous fish populations worldwide (Hutchings & Reynolds, 2004; Myers & Worm, 2003; Worm *et al*., 2006). Today, more than 75% of global fish stocks are fully exploited, overexploited or depleted (FAO, 2009). In South Africa, many linefish have been the target of overfishing, including kabeljou (dusky and silver kob), geelbek, red stumpnose, Roman seabream and carpenter seabream (Siebert, 2009). In an attempt to reverse the collapse in local fish stocks, the Southern African Sustainable Seafood Initiative (SASSI) was established in 2004 with the aim of educating and shifting consumer purchasing behaviour towards more sustainable seafood choices and to promote more sustainable management in the South African fishing industry. SASSI has developed and disseminated a sustainable seafood list which uses a traffic light system to rank seafood choices as green, orange or red, from the best to the worst choices (SASSI, 2010). However, a major limitation experienced globally with such awareness campaigns is that the recommendations made are only as effective as that information that is provided to consumers on the product labelling or by the vendors at the point of sale. If vital information on the species and conservation impact is not available, or if fish are mislabelled, poor choices may be inadvertently made in spite of the good intentions of the consumer (Jacquet & Pauly, 2007; 2008; Logan *et al*., 2008).

The aim of this study was to determine the most commonly available fish species in South Africa by means of restaurant and retail outlet surveys and to correlate these fish with the SASSI list in order to assess the sustainability of the prevailing fisheries market. Further aims were to assess the state in which commercially available fish were mostly sold (fresh, frozen, whole or filleted) and to evaluate the information available to consumers at the point of sale relating to the geographical origin, production method and sustainability of the fish products.

Materials and methods

Study and sampling design

The overall research design was to survey restaurants and retail outlets (supermarkets and fish markets) in South Africa to determine the most common fish species being sold on the domestic market. Restaurants and retail outlets were chosen for this purpose since they represent the main channels through which local consumers obtain fish products in this country (SeaFish, 2010), even though data are not currently available on the precise proportions of fish traded through each of these avenues. Four of the nine South African provinces were selected for the surveys. These included the three major fishing provinces of South Africa, namely KZN, WC and EC, and one inland province, namely GP (Fig. 1). The three aforementioned fishing provinces, listed in descending order in terms of their relative contributions to the South African TAC, were selected with the anticipation that these provinces would have a greater supply and availability of locally caught fish species compared with most inland provinces of South Africa. GP was included in the surveys as it is known to represent a principal market for seafood in South Africa, being the most populated province in the country with the highest per capita income (Schlemmer, 1998; SeaFish, 2010). A chi-square (χ^2) test power analysis (StatSoft Inc., 2009) was used to assess the sample size required to assess the availability of fish species in South African restaurants and retail outlets (Tables 1 and 2). Restaurants and retail outlets in the four provinces were identified and selected for the study prior to the initiation of surveys. Where possible, attempts were made to balance the sample sizes from high income and low income regions in each province. Surveys were carried out over a time period of one year (May 2009 to May 2010). Since data was collected in different provinces at different times of the year, seasonality may have affected the availability of fish in some regions of South Africa.

Fish availability - restaurants

A total of n = 215 restaurants were surveyed in South Africa, with n = 60 in the WC, n = 51 in the EC, n = 51 in KZN and n = 53 in GP. The basis for selection of restaurants for the surveys in each province was that the restaurant should have a dedicated seafood section on the menu and/or serve at least three different fish species. Interviews were conducted with managers of selected restaurants, who were asked to indicate which fish were routinely available on their respective menus, as well as which species were most commonly served as ‘linefish of the day’, ‘catch of the day’ or other ‘blackboard’ specials. Fish were recorded based on the names by which they were marketed in the restaurants and may not correlate in all cases with the expected common names (Froese & Pauly, 2010) for these species. The ability of restaurant managers to identify the fish being marketed was evaluated using the rating criteria listed in Table 3.

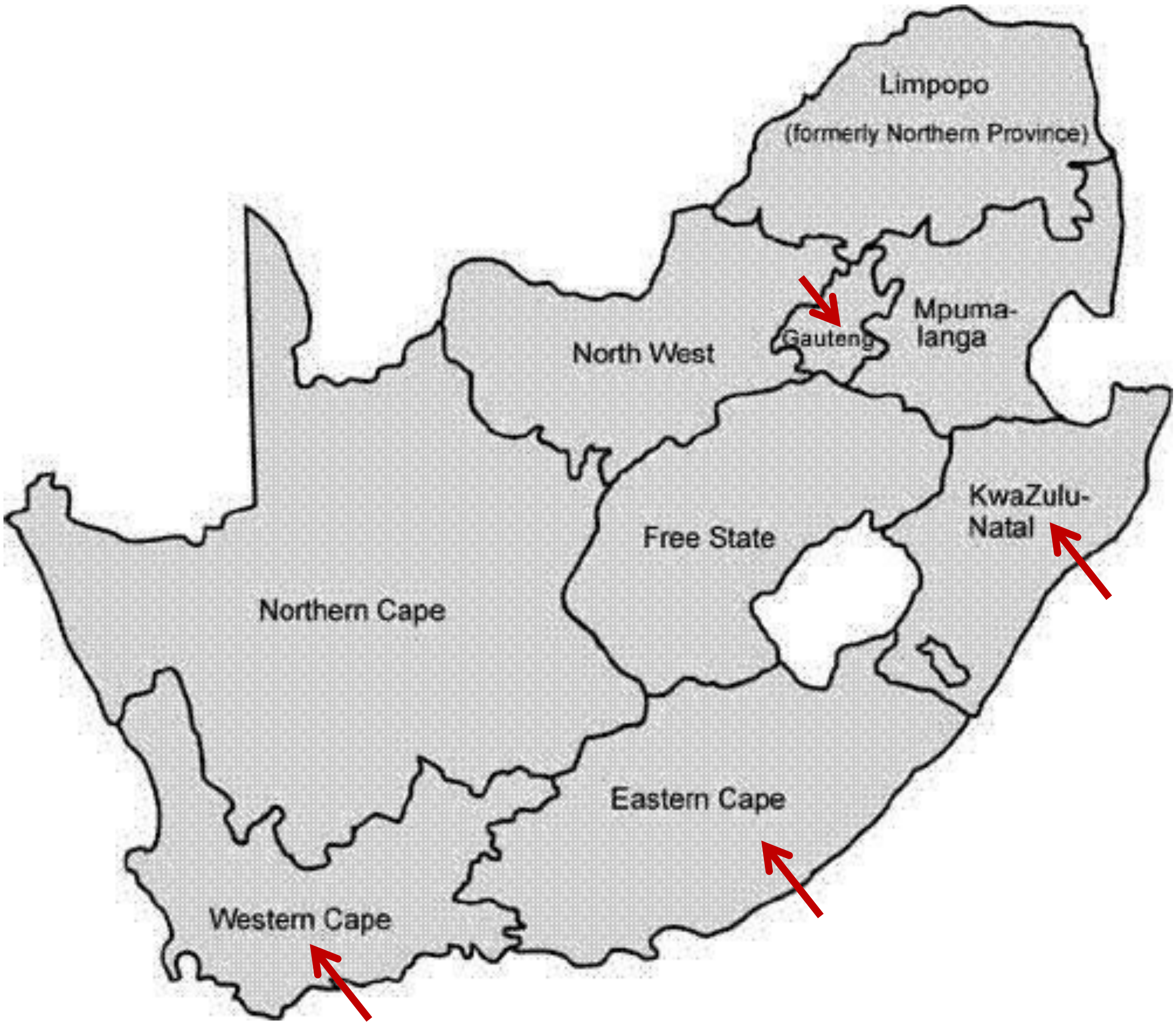


Figure 1 Map showing the nine provinces of South Africa with the four provinces surveyed in this study indicated with arrows.

Fish availability - retail outlets

A total of n = 200 retail outlets were surveyed in this study, which included n = 25 supermarkets and n = 25 fish markets in each of the four provinces. Supermarkets were defined as those stores that sold a range of food and grocery products, while fish markets were defined as those outlets selling primarily fish. In order to standardise the sampling protocol for supermarkets among the four provinces, five retail supermarket chains in South Africa were identified that market fresh and frozen fish products. The same numbers of each of these supermarkets were surveyed in each province. Fish markets were randomly selected in each province, based on the premise that they sold at least three different species of fish. At both the supermarkets and the fish markets, the types of packaged frozen fish and fresh fish were recorded. In the case where fish were sold unpackaged, vendors behind the counters were questioned on the identity of the available fish (if the fish were not labelled), and their ability to identify these fish was rated in accordance with the criteria listed in Table 3. When vendors were unable to provide the name for certain fish, whole fish were identified by visual inspection using keys and identification texts in hand. On packaged products, the market names of the fish provided and the Latin names (when available) were recorded. Where frozen products were sold under different market names, but represented the same species (such as herring and kippers which are both expected to be the species *Clupea herangus*), the products were assessed separately based on the market names. Canned and value-added fish products were not evaluated. Since recordings were based on whether a given species was available or not, the data presented in this study are not intended to indicate the relative abundance of each fish in terms of the number of different product types or the weights of the species marketed. Data from supermarkets and fish markets were combined for statistical analyses.

State and form in which fish were sold

For the assessment of the state in which fish types were predominantly sold on the retail market, the number of fresh and frozen products of given species were noted in supermarkets and fish markets, as was the proportion of fish sold whole or filleted.

Correlation of fish availability and sustainable seafood choices

The SASSI seafood list as available on 01 August 2010 (SASSI, 2010), was used to assess the sustainability status of all fish species observed in restaurants and retail outlets. In cases where specific fish species did not appear on the SASSI list, the

International Union for Conservation of Nature Red List (IUCN, 2010) was consulted to evaluate the conservation threat to the observed species.

Additional information on fish

Since one of the objectives of the study was to assess the information available to consumers regarding the fish they select, restaurant managers and vendors at fresh fish counters in retail outlets were questioned on the geographical origin of each species they marketed and on whether the fish were wild caught or farmed. Interviewees where also asked whether they could indicate which of their available fish were good choices in terms of sustainability and which were of conservation concern. The knowledge of restaurant managers and vendors in terms of the aforementioned parameters was rated using the criteria listed in Table 3. Additionally, all information provided on the labels of packaged products in retail outlets was noted relating to the geographical origin, production method and sustainability of the enclosed fish (Table 4).

Statistical analysis

All statistical analyses were performed using Statistica™ 9.0 (StatSoft Inc., 2009). The maximum-likelihood (M-L) chi-square test (χ^2) was used to compare the availability of fish species as a function of location (province in which observed), outlet (restaurant or retail outlet) and the processing state in which they were marketed (fresh, frozen, whole, filleted). P-values of <0.05 were considered to be statistically significant.

Results and discussion

Fish availability - restaurants

The observed availability of different fish types in restaurants in each province is shown in Table 1, while the total availability of fish types in all surveyed restaurants (n = 215) is shown in Figure 2. On average, the widest variety of fish species available per restaurant was found in the WC, followed by GP and KZN and then EC (Table 1). A total of 34 nominal types of fish were found to be marketed in the restaurants surveyed in all four provinces (Table 1). Only three of these fish types were available in more than 50% of the total restaurants (Fig. 2). Kingklip was the most commonly available fish species in all 215 restaurants, occurring in 88% of the outlets surveyed, followed by Atlantic salmon (59% occurrence) and hake (58% occurrence) (Fig. 2). Certain fish, such as white steenbras and white stumpnose, occurred sporadically in restaurants and only in one or two of the provinces. For the simplicity of graphical representations, fish occurring in less than 1% of the total restaurants are not included in Figure 2.

For many of the observed fish types, the results of the chi-square test revealed strong evidence against the null hypothesis of no regional differences in the availability of fish in restaurants. *P*-values of ≤ 0.05 indicate that a given species was not equally available in restaurants in all provinces (Table 1). Overall, there was no significant difference ($p > 0.05$) among provinces in the availability of 12 of the 34 fish (35%) in restaurants, while the availability of the remaining 22 (65%) of the observed fish types appeared to differ between provinces ($p \leq 0.05$). Of the most commonly observed fish in restaurants, kingklip and hake appeared to be similarly available in all four provinces, as was the availability of east coast sole, west coast sole and butterfish ($p > 0.05$). Atlantic salmon, yellowfin tuna and kabeljou were more commonly available in GP and WC than they were in EC and KZN ($p \leq 0.05$) (Table 1). Fish such as yellowtail, geelbek (Cape salmon), angelfish, trout and snoek were most commonly available in the WC, while dorado and Roman seabream were most popular in KZN. Certain fish were observed to be exclusively available in the restaurants of only one surveyed province. For instance, yellowbelly and catface/spotted rockcods were only observed in the restaurants of KZN, as was couta and Natal snoek. On the other hand, gurnard was only available in EC restaurants, trout and bluenose only in WC restaurants and the fish marketed as 'red snapper' was only available in GP restaurants (Table 1).

Fish availability - retail outlets

The observed availability of fish species in retail outlets in each province is shown in Table 2, while the total availability of fish in all 200 retail outlets (n = 100 supermarkets and n = 100 fish markets) is graphically represented in Figure 3. On average, the WC had the greatest variety of different fish types per retail outlet, with the availability being similar in supermarkets and fish markets (Table 2). An unexpected finding was that, as seen in the restaurant surveys, the inland province of GP had the second largest variety of fish species available per retail outlet. Prior to the study, it had been expected that the retail outlets located along the coast of South Africa would have the greatest access to locally caught fish and would thus be likely to have the largest variety of species available. In fact, the results of the retail outlet surveys revealed that the species diversity in GP was greater per outlet than that observed in the coastal provinces of KZN and EC.

Table 1 Fish availability by frequency of species appearance in restaurants in four provinces of South Africa

Market name	Expected species (scientific name)	WC	EC	KZN	GP	χ^2 (p)	SASSI list
Number of restaurants		60	51	51	53		
Number of fish observed in all restaurants		352	225	250	276		
Mean number of fish per restaurant (rounded)		6	4	5	5		
Kingklip	*Genypterus capensis*	90.0%	90.2%	86.0%	84.9%	1.1 (0.77)	Orange
Atlantic salmon	*Salmo salar*	61.7%	51.0%	37.3%	84.8%	28.1 (<0.01)	NE [LC]
Hake	*Merluccius paradoxus* / *Merluccius capensis*	63.3%	58.8%	62.8%	45.3%	4.7 (0.20)	Green
East coast sole	*Austroglossus pectoralis*	45.0%	58.8%	43.1%	50.9%	3.1 (0.37)	Orange
Yellowfin tuna	*Thunnus albacares*	53.3%	29.4%	23.5%	62.3%	22.9 (<0.01)	Green
Dorado	*Coryphaena hippurus*	36.7%	19.6%	62.8%	24.5%	24.8 (<0.01)	Green
Kabeljou / kob	*Argyrosomus* spp.	33.3%	9.8%	13.7%	39.2%	19.1 (<0.01)	Orange
West coast sole	*Austroglossus microlepis*	31.7%	27.5%	11.8%	22.6%	7.1 (0.07)	Orange
Yellowtail	*Seriola lalandi*	33.3%	13.7%	5.9%	20.8%	15.4 (<0.01)	Green
Butterfish	*Ruvettus pretiosus* / *Lepidocybium flavobrunneum*	23.3%	19.6%	15.7%	11.3%	3.1 (0.37)	Green
Geelbek / Cape salmon	*Atractoscion aequidens*	30.0%	11.7%	9.8%	11.3%	10.6 (0.01)	Orange
Angelfish	*Brama brama*	21.7%	3.9%	13.7%	3.8%	13.0 (<0.01)	Green
Sardine / pilchard	*Sardinops sagax*	6.7%	5.9%	7.8%	17.0%	4.5 (0.21)	Green
Swordfish	*Xiphias gladius*	10.0%	0.0%	7.8%	7.6%	8.2 (0.04)	Orange
Roman	*Chrysoblephus laticeps*	1.7%	3.9%	19.6%	0.0%	20.6 (<0.01)	Orange
Codfish	*Gadus morhua*	1.7%	2.0%	2.0%	13.2%	9.7 (0.02)	NE [V]
Silverfish / carpenter	*Argyrozona argyrozona*	5.0%	9.8%	2.0%	0.0%	8.4 (0.04)	Orange
Trout	*Oncorhynchus mykiss*	10.0%	2.0%	0.0%	0.0%	12.9 (<0.01)	NE [NE]
Yellowbelly rockcod	*Epinephelus marginatus*	0.0%	0.0%	13.7%	0.0%	20.9 (<0.01)	Orange
Snoek	*Thyrsites atun*	8.3%	0.0%	0.0%	1.9%	10.4 (0.02)	Green
Santer / soldier	*Cheimerius nufar*	0.0%	5.9%	5.9%	0.0%	9.1 (0.02)	Green
Gurnard	*Chelidonichthys* spp.	0.0%	11.8%	0.0%	0.0%	17.8 (<0.01)	Green
Marlin	*Makaira* / *Tetrapturus* spp.	3.3%	0.0%	5.9%	0.0%	7.1 (0.07)	Orange
Catface/spotted rockcod	*Epinephulus andersoni*	0.0%	0.0%	9.8%	0.0%	14.8 (<0.01)	Orange
Couta / King mackerel	*Scomberomorus commerson*	0.0%	0.0%	9.8%	0.0%	14.8 (<0.01)	Orange
Natal snoek / Queen mackerel	*Scomberomorus plurilineatus*	0.0%	0.0%	9.8%	0.0%	14.8 (<0.01)	Green
Panga	*Pterogymnus laniarus*	0.0%	2.0%	2.0%	5.7%	4.8 (0.19)	Green
Dory	*Zeus* spp.	3.3%	2.0%	3.9%	0.0%	3.2 (0.36)	Green
Cardinal	*Epigonus telescopus*	3.3%	0.0%	3.9%	0.0%	5.4 (0.15)	NE [NE]
Red snapper	*Lutjanus* spp.	0.0%	0.0%	0.0%	5.7%	8.5 (0.04)	NE / {R}
Anchovy	*Engraulis* spp.	1.7%	0.0%	2.0%	1.9%	1.7 (0.65)	Green
Bluenose	*Hyperoglyphe antarctica*	5.0%	0.0%	0.0%	0.0%	7.8 (0.05)	Green
White steenbras	*Lithognathus lithognathus*	1.7%	0.0%	2.0%	0.0%	2.7 (0.44)	Red
White stumpnose	*Rhabdosargus globiceps*	1.7%	0.0%	2.0%	0.0%	2.7 (0.44)	Green

NE = Not evaluated by SASSI; {R} = certain members of the genus are on the SASSI red list; [NE] = Not evaluated by International Union for Conservation of Nature (IUCN), [LC] = Least concern according to IUCN, V = vulnerable according to IUCN.

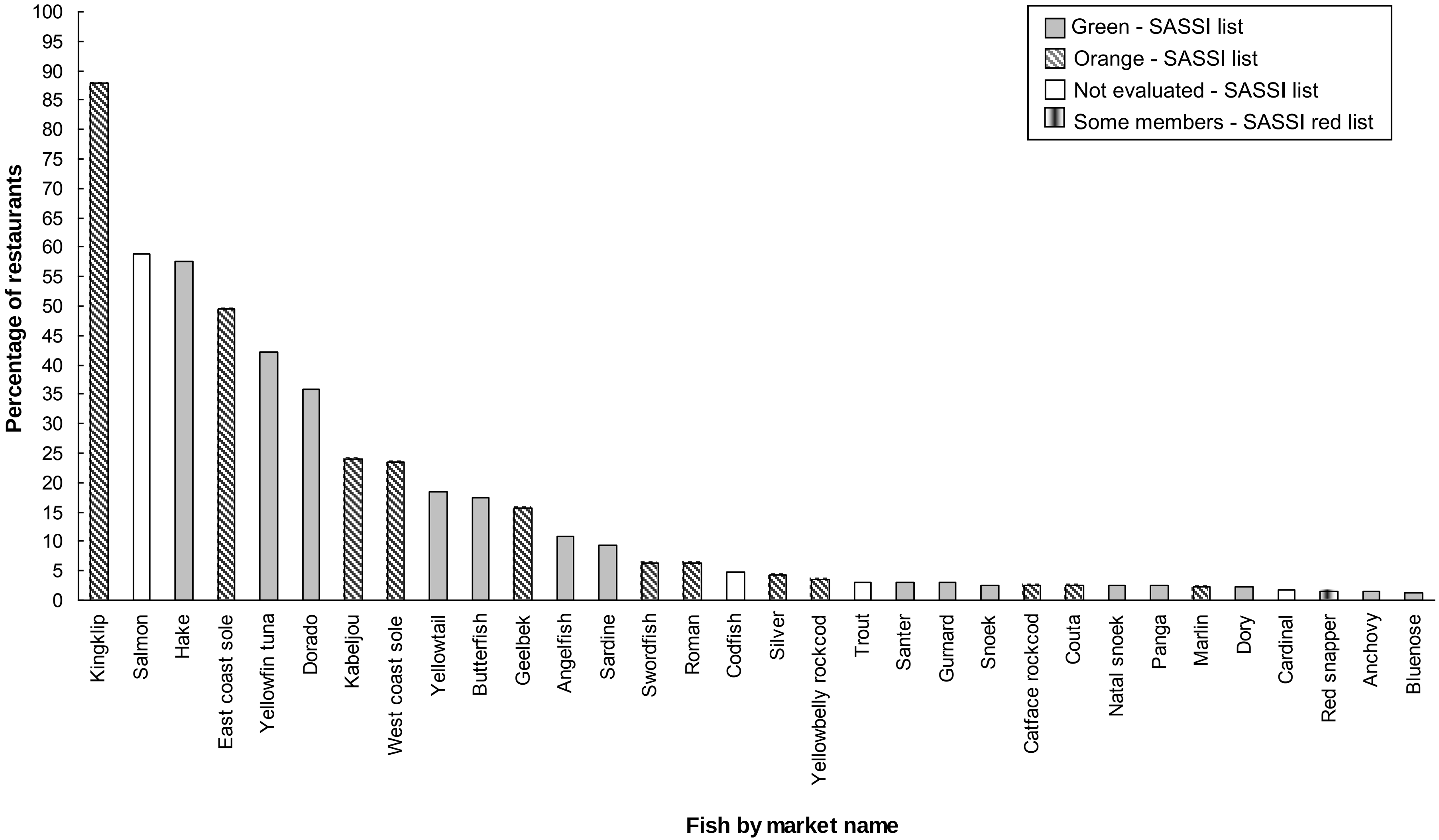


Figure 2 The total observed availability of fish by the frequency of species appearance combining the data from restaurants in four provinces (n = 215) in South Africa. Fish species available in less than one percent of the total restaurants are excluded from the graphical representation. The conservation statuses of fish are classified according to the SASSI list as available on 01 August 2010.

Table 2 Fish availability by frequency of species appearance in retail outlets (supermarkets and fish markets) in four provinces of South Africa

Market name	**Expected species (scientific name)**	**WC**	**EC**	**KZN**	**GP**	**χ^2 (p)**	**SASSI list**
Number of retail outlets (supermarkets/fish markets)		50 (25/25)	50 (25/25)	50 (25/25)	50 (25/25)		
Number of fish observed in all outlets (supermarkets/fish markets)		580 (294/286)	398 (242/156)	403 (197/206)	492 (296/196)		
Mean number of fish per retail outlet (supermarkets/fish markets)		12 (12/11)	8 (10/6)	8 (8/8)	10 (12/8)		
Hake	*Merluccius paradoxus* / *Merluccius capensis*	100.0%	98.0%	78.0%	90.0%	21.3 (<0.01)	Green
Kingklip	*Genypterus capensis*	82.0%	74.0%	56.0%	88.0%	15.2 (<0.01)	Orange
Snoek	*Thyrsites atun*	94.0%	68.0%	54.0%	50.0%	31.4 (<0.01)	Green
Atlantic salmon	*Salmo salar*	66.0%	50.0%	46.0%	80.0%	15.9 (<0.01)	NE [LC]
Haddock	*Merluccius* spp.	58.0%	56.0%	46.0%	48.0%	2.1 (0.55)	Green
Yellowfin tuna	*Thunnus albacares*	52.0%	32.0%	32.0%	62.0%	13.8 (<0.01)	Green
East coast sole	*Austroglossus pectoralis*	60.0%	44.0%	26.0%	46.0%	12.2 (<0.01)	Orange
Kippers	*Clupea herangus*	42.0%	40.0%	34.0%	36.0%	0.9 (0.84)	NE [NE]
Yellowtail	*Seriola lalandi*	50.0%	28.0%	6.0%	40.0%	29.0 (<0.01)	Green
Mackerel	*Scomber japonicus*	40.0%	24.0%	24.0%	34.0%	4.4 (0.22)	Green
Cape whiting	*Merluccius* spp.	30.0%	30.0%	26.0%	26.0%	0.4 (0.94)	Green
Kabeljou / kob	*Argyrosomus* spp.	28.0%	24.0%	18.0%	28.0%	1.9 (0.60)	Orange
Dorado	*Coryphaena hippurus*	20.0%	8.0%	32.0%	32.0%	12.4 (<0.01)	Green
Butterfish	*Ruvettus pretiosus* / *Lepidocybium flavobrunneum*	26.0%	20.0%	16.0%	28.0%	2.7 (0.45)	Green
Silver / carpenter	*Argyrozona argyrozona*	24.0%	28.0%	14.0%	16.0%	4.0 (0.26)	Orange
Angelfish	*Brama brama*	32.0%	8.0%	18.0%	20.0%	9.6 (0.02)	Green
Santer / soldier	*Cheimerius nufar*	10.0%	14.0%	30.0%	14.0%	7.8 (0.05)	Green
Geelbek / Cape salmon	*Atractoscion aequidens*	22.0%	18.0%	10.0%	18.0%	2.9 (0.41)	Orange
Roman	*Chrysoblephus laticeps*	14.0%	4.0%	28.0%	18.0%	12.1 (<0.01)	Orange
West coast sole	*Austroglossus microlepis*	30.0%	8.0%	8.0%	16.0%	11.7 (<0.01)	Orange
Maasbanker / Cape horse mackerel	*Trachurus capensis*	22.0%	18.0%	14.0%	6.0%	6.1 (0.11)	Green
Trout	*Oncorhynchus mykiss*	20.0%	4.0%	8.0%	20.0%	9.8 (0.02)	NE [NE]
Monkfish	*Lophius vomerinus*	18.0%	6.0%	8.0%	18.0%	5.9 (0.12)	Green
Portugese sardine	*Sardina pilchardus*	22.0%	4.0%	6.0%	12.0%	9.7 (0.02)	NE [NE]
Herring	*Clupea herangus*	12.0%	12.0%	6.0%	14.0%	2.0 (0.57)	NE [NE]
Gurnard	*Chelidonichthys* spp.	16.0%	22.0%	2.0%	2.0%	18.1 (<0.01)	Green
Panga	*Pterogymnus laniarus*	16.0%	8.0%	4.0%	12.0%	4.7 (0.20)	Green
Sardine / pilchard	*Sardinops sagax*	18.0%	6.0%	4.0%	2.0%	10.1 (0.02)	Green
Catface/spotted rockcod	*Epinephulus andersoni*	0.0%	0.0%	12.0%	12.0%	18.1 (<0.01)	Orange
Slinger	*Chrysoblephus puniceus*	0.0%	0.0%	24.0%	2.0%	31.3 (<0.01)	Orange
White stumpnose	*Rhabdosargus globiceps*	8.0%	8.0%	4.0%	4.0%	1.4 (0.70)	Green
Yellowbelly rockcod	*Epinephelus marginatus*	0.0%	0.0%	12.0%	8.0%	14.8 (<0.01)	Orange
Red snapper	*Lutjanus* spp.	2.0%	0.0%	2.0%	18.0%	18.5 (<0.01)	NE / {R}
Buttersnoek	*Lepidopus caudatus*	12.0%	0.0%	8.0%	0.0%	14.8 (<0.01)	Green

Table 2 (continued)

Market name	Expected species (scientific name)	WC	EC	KZN	GP	χ^2 (p)	SASSI list
Jacopever	*Helicolenus dactylopterus*	12.0%	2.0%	4.0%	2.0%	6.3 (0.10)	Green
Hottentot	*Pachymetopon blochii*	12.0%	2.0%	2.0%	4.0%	6.3 (0.10)	Green
Swordfish	*Xiphias gladius*	8.0%	0.0%	6.0%	4.0%	6.0 (0.11)	Orange
Red stumpnose	*Chrysoblephus gibbiceps*	2.0%	4.0%	2.0%	6.0%	1.6 (0.66)	Orange
White musselcracker	*Sparodon durbanensis*	0.0%	4.0%	8.0%	0.0%	9.2 (0.03)	Red
Couta / King mackerel	*Scomberomorus commerson*	0.0%	0.0%	12.0%	0.0%	17.2 (<0.01)	Orange
Dory	*Zeus* spp.	6.0%	2.0%	2.0%	2.0%	1.8 (0.62)	Green
Longfin tuna	*Thunnus alalunga*	8.0%	0.0%	0.0%	4.0%	9.2 (0.03)	Green
Hoki hake	*Macruronus Magellanicus*	8.0%	0.0%	0.0%	4.0%	9.2 (0.03)	NE [NE]
Alaskan (chum) salmon	*Oncorhynchus keta*	6.0%	0.0%	0.0%	6.0%	8.5 (0.04)	NE [NE]
Barramundi	*Lates calcarifer*	4.0%	4.0%	2.0%	2.0%	0.7 (0.87)	NE [NE]
Skipjack tuna	*Katsuwonus pelamis*	4.0%	0.0%	4.0%	2.0%	3.4 (0.34)	Green
White steenbras	*Lithognathus lithognathus*	0.0%	2.0%	8.0%	0.0%	9.1 (0.03)	Red
Englishman	*Chrysoblephus anglicus*	0.0%	0.0%	4.0%	6.0%	7.3 (0.06)	Orange
Bluenose	*Hyperoglyphe antarctica*	8.0%	0.0%	0.0%	2.0%	9.1 (0.03)	Green
Yellowfin sole	*Limanda aspera*	8.0%	0.0%	0.0%	2.0%	9.1 (0.03)	NE [NE]
Natal snoek / Queen mackerel	*Scomberomorus plurilineatus*	0.0%	0.0%	6.0%	0.0%	8.5 (0.04)	Green
Marlin	*Makaira / Tetrapturus* spp.	4.0%	0.0%	2.0%	0.0%	4.6 (0.21)	Orange
Blue warehou	*Seriolella brama*	0.0%	0.0%	2.0%	4.0%	4.6 (0.21)	NE [NE]
Tomato rockcod	*Cephalopholis sonnerati*	0.0%	0.0%	4.0%	0.0%	5.6 (0.13)	Orange
Wahoo	*Acanthocybium solandri*	0.0%	0.0%	4.0%	0.0%	5.6 (0.13)	NE [NE]
Sailfish	*Istiophorus* spp.	4.0%	0.0%	0.0%	0.0%	5.6 (0.13)	Orange
Jack mackerel	*Trachurus* spp.	2.0%	0.0%	0.0%	2.0%	2.8 (0.43)	NE [NE]
Anchovy	*Engraulis* spp.	4.0%	0.0%	0.0%	0.0%	5.6 (0.13)	Green
Butter bream	*Monodactylus argenteus*	0.0%	4.0%	0.0%	0.0%	5.6 (0.13)	NE [NE]
Barracuda	*Sphyraena barracuda*	0.0%	0.0%	2.0%	2.0%	2.8 (0.43)	NE [NE]
Black ruff	*Centrolophus niger*	0.0%	0.0%	0.0%	4.0%	5.6 (0.13)	NE [NE]
Scavenger/emperor	*Lethrinus* spp.	0.0%	0.0%	2.0%	2.0%	2.8 (0.43)	NE [NE]
Natal stumpnose	*Rhabdosargus sarba*	0.0%	0.0%	2.0%	0.0%	2.8 (0.43)	Red
Halfmoon rockcod	*Epinephelus rivulatus*	0.0%	0.0%	2.0%	0.0%	2.8 (0.43)	Orange
Black musselcracker	*Cymatoceps nasutus*	0.0%	0.0%	2.0%	0.0%	2.8 (0.43)	Orange
Basa	*Pangasius bocourti*	2.0%	0.0%	0.0%	0.0%	2.8 (0.43)	NE [NE]
Kahawai	*Arripis trutta*	2.0%	0.0%	0.0%	0.0%	2.8 (0.43)	NE [NE]
Codfish	*Gadus morhua*	2.0%	0.0%	0.0%	0.0%	2.8 (0.43)	NE [V]
Dageraad	*Chrysoblephus cristiceps*	0.0%	2.0%	0.0%	0.0%	2.8 (0.43)	Orange
Red steenbras	*Petrus rupestris*	0.0%	0.0%	0.0%	2.0%	2.8 (0.43)	Orange

NE = Not evaluated by SASSI; {R} = certain members of the genus are on the SASSI red list; [NE] = Not evaluated by International Union for Conservation of Nature (IUCN), [LC] = Least concern according to IUCN, [V] = vulnerable according to IUCN.

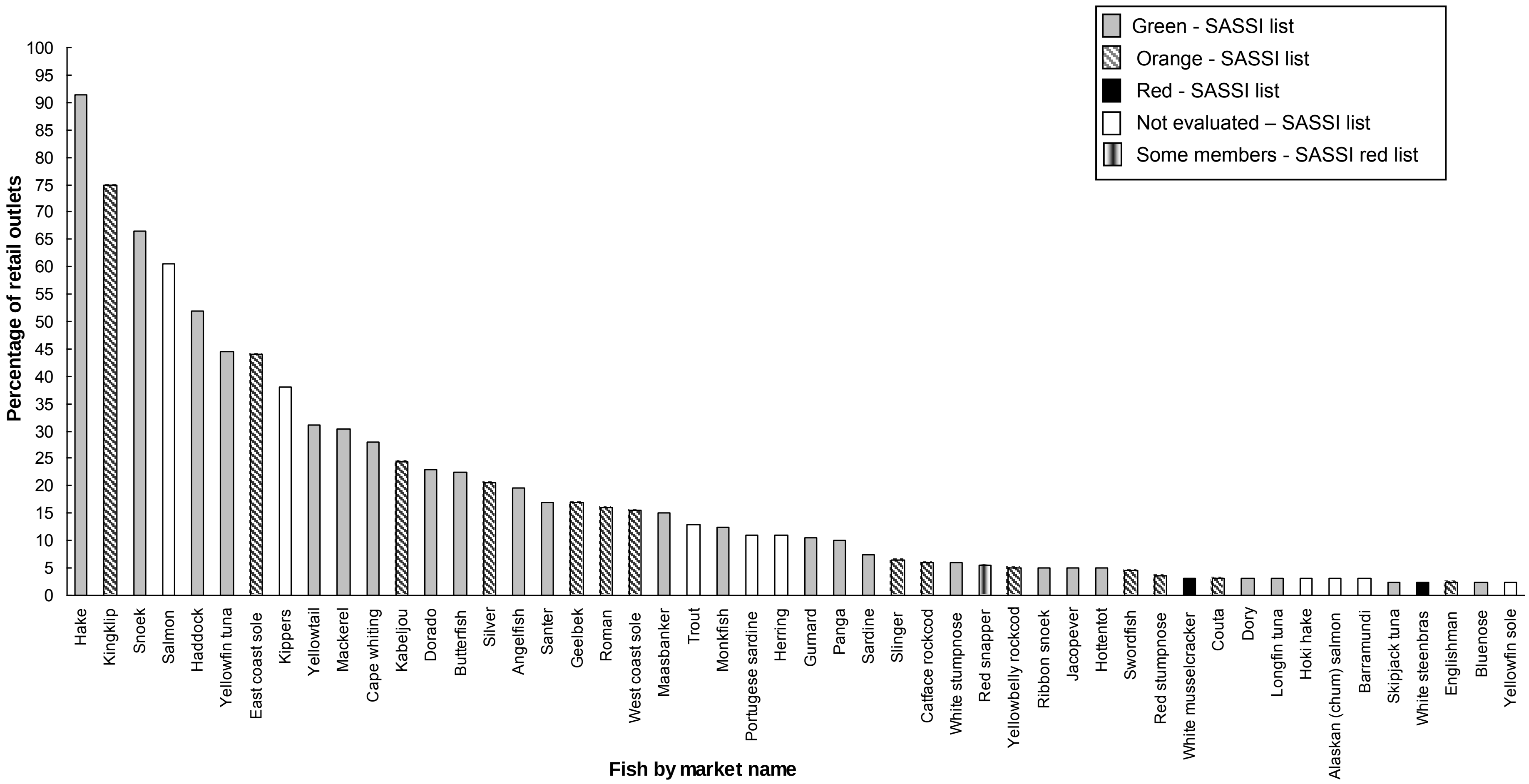


Figure 3 The total observed availability of fish by the frequency of appearance combining the data from retail outlets (supermarkets and fish markets) in four provinces (n = 200) in South Africa. Fish species available in two percent or less of the total retail outlets are excluded from the graphical representation. The conservation statuses of fish are classified according to the SASSI list as available on 01 August 2010.

Seventy nominal types of fish were observed in the 200 retail outlets surveyed in all four provinces of South Africa (Table 2). Five of these fish types were available in more than 50% of the total retail outlets (Fig. 3). Hake was the most commonly observed fish species in all 200 retail outlets, occurring in 92% of the outlets surveyed, followed by kingklip (75% occurrence) and snoek (67% occurrence) (Fig. 3). However, it should be noted that while these three fish were observed to be most common by the frequency of species appearance, the number of different hake products being marketed (particularly in supermarkets) far exceeded the number of different kingklip and snoek products. In addition, hake is often marketed in South Africa as haddock or Cape Whiting (Table 2), and while this may be confusing or not always apparent to the public, it was found that the relative abundance of *Merluccius* species on the South African retail market far exceeded any other fish type observed in this study. A great number of fish, such as Natal stumpnose, black musselcracker (poenskop), dageraad and red steenbras occurred sporadically in retail outlets and only in one or two of the surveyed provinces. For the simplicity of graphical representations, fish occurring in less than 2% of the total retail outlets are not included in Figure 3.

As was the case with the restaurants surveyed, the null hypothesis of no regional differences in the availability of fish in retail outlets could not be accepted with all fish observed. Overall, 40 of the 70 (57%) observed fish types appeared to have a similar availability among provinces ($p > 0.05$), while the remainder appeared to differ between provinces ($p \leq 0.05$). The difference in the availability of certain fish between provinces was generally observed to be as a result of the diverse range of fish products being marketed in fish markets. Of the most commonly observed fish in retail outlets, the availability of hake and kingklip was found to differ among provinces ($p \leq 0.05$), both being less commonly available in the retail outlets of KZN.

As was seen in the restaurant surveys (Table 1), Atlantic salmon and yellowfin tuna were more commonly available in GP and WC than they were in EC and KZN ($p \leq 0.05$). Snoek, yellowtail and angelfish were again observed to be the most popular in the WC (Table 2). Certain fish were observed to be most commonly or exclusively available in the retail outlets of only one surveyed province. For instance, Roman, slinger and santer (called soldier in KZN) were most commonly available in KZN, while couta, Natal snoek, tomato and halfmoon rockcods, Natal stumpnsoe and black musselcracker were found only in the retail outlets of this province. As was the case in restaurants, the fish marketed as ‘red snapper’ was predominantly available in the retail outlets of GP (Table 2).

State and form in which fish were sold

The percentages of the observed fish available in retail outlets that were marketed fresh or frozen and whole or filleted are shown in Figure 4A and Figure 4B, respectively. The null hypotheses of no regional differences in the proportions of fresh and frozen fish, as well as the proportions of whole or filleted fish, were generally accepted from the results of the chi-square tests. That is to say that all four provinces had similar proportions of fresh and frozen fish species and whole and filleted fish species available in retail outlets. Of the total number of different fish products observed, supermarkets had a greater number of fresh and frozen fish products available than fish markets. Supermarkets also had a greater number of different filleted fish products available than fish markets, but fish markets had a greater number of whole fish than supermarkets. In total, taking all four provinces into account, 42% of the different fish products in the surveyed retail outlets were marketed fresh and 58% were marketed frozen. Overall, 32% of the fish products sold in all four provinces were sold whole (mostly head on and viscera removed) and 68% were sold in their filleted form. Figure 5 shows the relationship between the processing states of fish (fresh or frozen) and the form in which these products were sold (whole or filleted) in retail outlets in all four provinces, separately and combined. Inspection of this figure reveals that, overall, approximately 70% of the whole fish observed in retail outlets were available in the fresh state, while the remaining 30% were marketed frozen. In addition, approximately 71% of all filleted fish products were sold frozen and the remaining 29% of filleted fish were sold in the fresh form. This pattern in the proportions of fresh and frozen fillets and whole fish was similar in all four provinces.

Fish availability and sustainability

Of the 34 fish types observed in restaurants and the 70 fish types observed in retail outlets, only 16 (47%) and 26 (37%), respectively, were listed on the SASSI green list (Tables 1 and 2). According to the SASSI classification of South African fish, green-listed species are those from relatively healthy, well-managed fish populations that are likely to handle current fishing pressures (SASSI, 2010). It was found that 12 of the 34 fish (35%) marketed in restaurants and 21 of the 70 fish (30%) marketed in retail outlets were on the SASSI orange list, the most popular of which were kingklip, east coast sole, west coast sole, kabeljou and geelbek. Orange-listed species are those that are currently overexploited, vulnerable to overfishing or which are caught using environmentally problematic methods. When taking only those fish species into account which were available in more than 15% of the restaurants and retail outlets (Figs 1 and 2), 50% of the observed restaurant species and nearly 40% of the retail species were SASSI orange-list species. While it is recommended by SASSI that orange-list species should to be considered with caution, it was apparent that many of the fish that are currently regarded as endangered are also those that are the most popular on the South African market.

Kingklip, for instance, appears on the SASSI orange list, but was found to feature in *ca.* 88% of the restaurants and 75% of the retail outlets surveyed (Figs 2 and 3). In the 1980s, an experimental longline fishery in South Africa considerably depleted kingklip stocks and the stocks remain reduced to this day (McLean & Glazewski, 2009). The TAC for kingklip in South Africa is only about 3 000 tons per annum, compared to that of approximately 130 000 tons for hake (DEAT, 2005). As far back as the 1960s, it was realized that kingklip is by no means abundant and that “far more fish named ‘kingklip’ on menus is eaten than is ever caught” (Smith & Smith, 1966). Thus, it has been questioned whether all the kingklip on the market is legally caught and/or whether it is even South African kingklip at all. A closely-related species known as ling or pink cusk eel (*Genypterus blacodes*) can be imported from Australia, New Zealand or South America for a lower cost than that required to obtain local kingklip, which also means that it can be sold as kingklip on the market for a greater profit (Bega, 2007).

Another conservation issue may surround the widespread occurrence of soles of the *Austroglossus* spp. in South Africa, with the east coast sole being found to be over 2-fold more commonly available (41% occurrence) in restaurants and retail outlets than the closely-related west coast sole (18% occurrence) (Fig 3). In recent years, catches of west coast sole have greatly decreased (Van der Elst, 1997) and the formal-directed fishery for this species has collapsed. East coast sole, caught predominately by a species-directed fishery from Mossel Bay in South Africa, has also come under increasing fishing pressure in recent years. Nonetheless, the real conservation issue surrounding this species relates to the destructive fishing methods used for its capture, which generate large bycatches of a variety of endangered South African linefish, frequently including juvenile specimens of the overexploited kabeljou/kob species (SASSI, 2010).

On the South African market, ‘kabeljou’ or ‘kob’ comprise three species, which all belong to the genus *Argyrosomus* and which were previously thought to be a single species (Griffiths & Heemstra, 1995). The stocks of all three species (silver kob, dusky kob and squaretail kob) are considered to be overexploited in South Africa (Griffiths,

2000) and are listed on the SASSI orange list (SASSI, 2010). Stocks of kabeljou are reportedly currently at less than 5% of their original breeding stock biomass (Siebert, 2009). However, the conservation issues surrounding kabeljou probably remain unknown to many South African consumers, since almost 25% of the restaurants and retail outlets combined marketed members of these species. Similarly, the orange-listed geelbek (Cape salmon) was available in 17% of the total outlets surveyed, yet the stocks of this species are severely overexploited and require rebuilding through more stringent regulations (SASSI, 2010).

From both a sustainability and regulatory viewpoint, perhaps one of the most startling findings emerging from this study was the observation of red-list species being actively sold on the South African market. Red-listed fish are those that are illegal to buy or sell in South Africa as these are recreational or specially protected species (SASSI, 2010). White steenbras (pignose grunter), a SASSI red-list species, was observed being marketed in restaurants in the WC and KZN (Table 1), as well as in retail outlets in the EC and KZN (Table 2). Stocks of this species are considered collapsed and the fish has been deemed as specially protected and is prohibited for sale in South Africa. This legal provision appeared to have little bearing on the open promotion of this species on the market by ignorant or dishonest vendors. It is, however, recognised by SASSI (2010) that many fish in South Africa may be misnamed as white steenbras when being sold to the public. In this study, 'white steenbras' was most often marketed in the filleted form, and it was therefore not possible in most cases to assess visually whether the fish were, in fact, white steenbras or not. Nonetheless, the marketing of endangered or red-list species, even in the case that they are not what they are said to be, hides the true status of the stocks, creating the false perception to consumers that the prevailing supplies are able to keep up with market demand. In addition to white steenbras, other red-list species such as white musselcracker and Natal stumpnose were also observed being flouted in retail markets, mainly in KZN (Table 2). Both white musselcracker and Natal stumpnose are important recreational species and have been designated as such as these species cannot withstand the pressure of commercial fishing.

Information on non-packaged fish in restaurants and retail outlets

Table 3 shows the perceived ability of managers in restaurants and vendors selling fresh non-packaged fish at retail counters to provide information to consumers about the fish that they were selling, assessed using the rating criteria presented in this table.

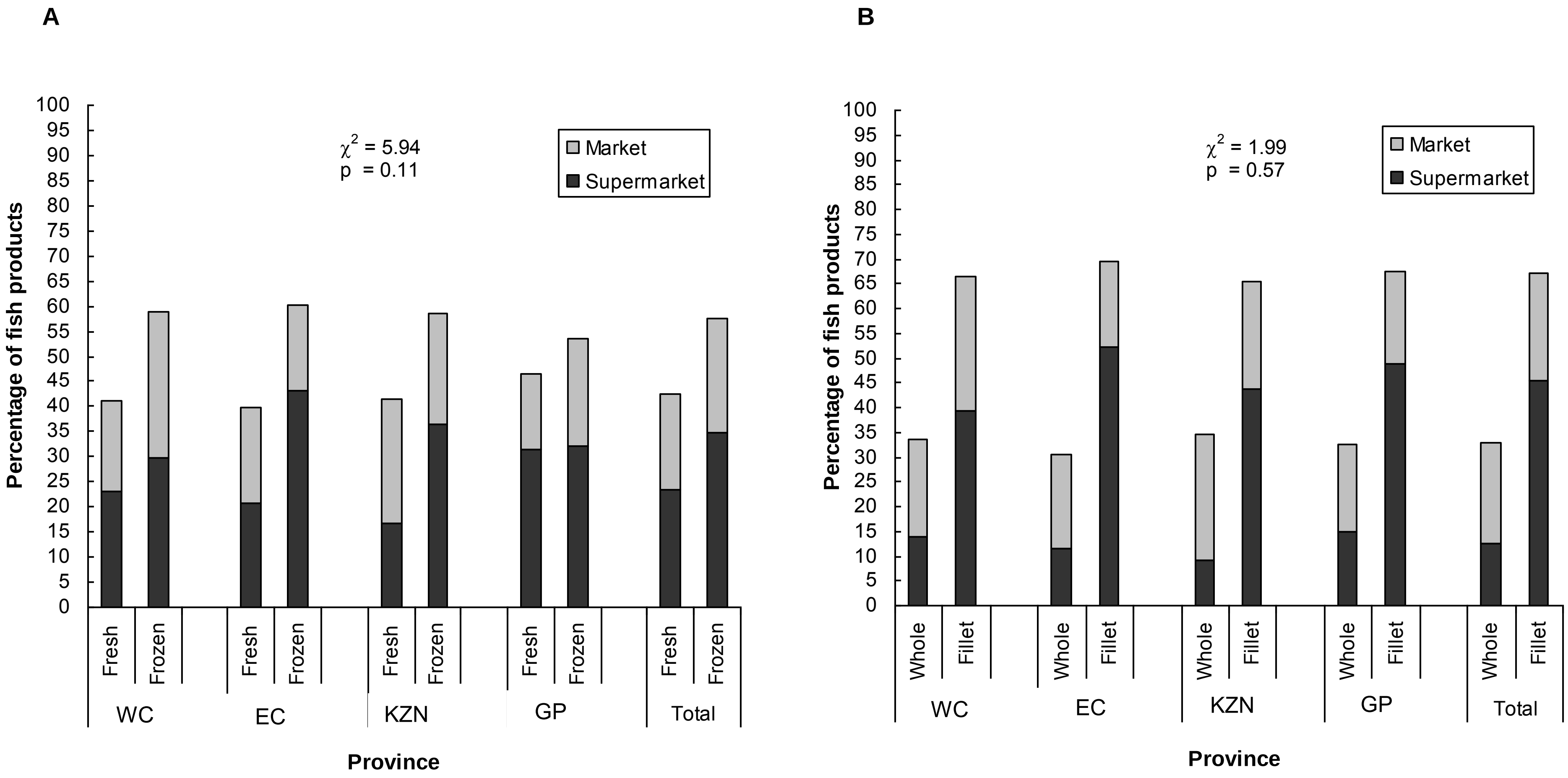


Figure 4 The percentage of fresh and frozen fish products (A) and whole and filleted fish products (B) available in retail outlets per province (n = 50 outlets per province) evaluated in South Africa, showing the relative proportions observed in fish markets and supermarkets.

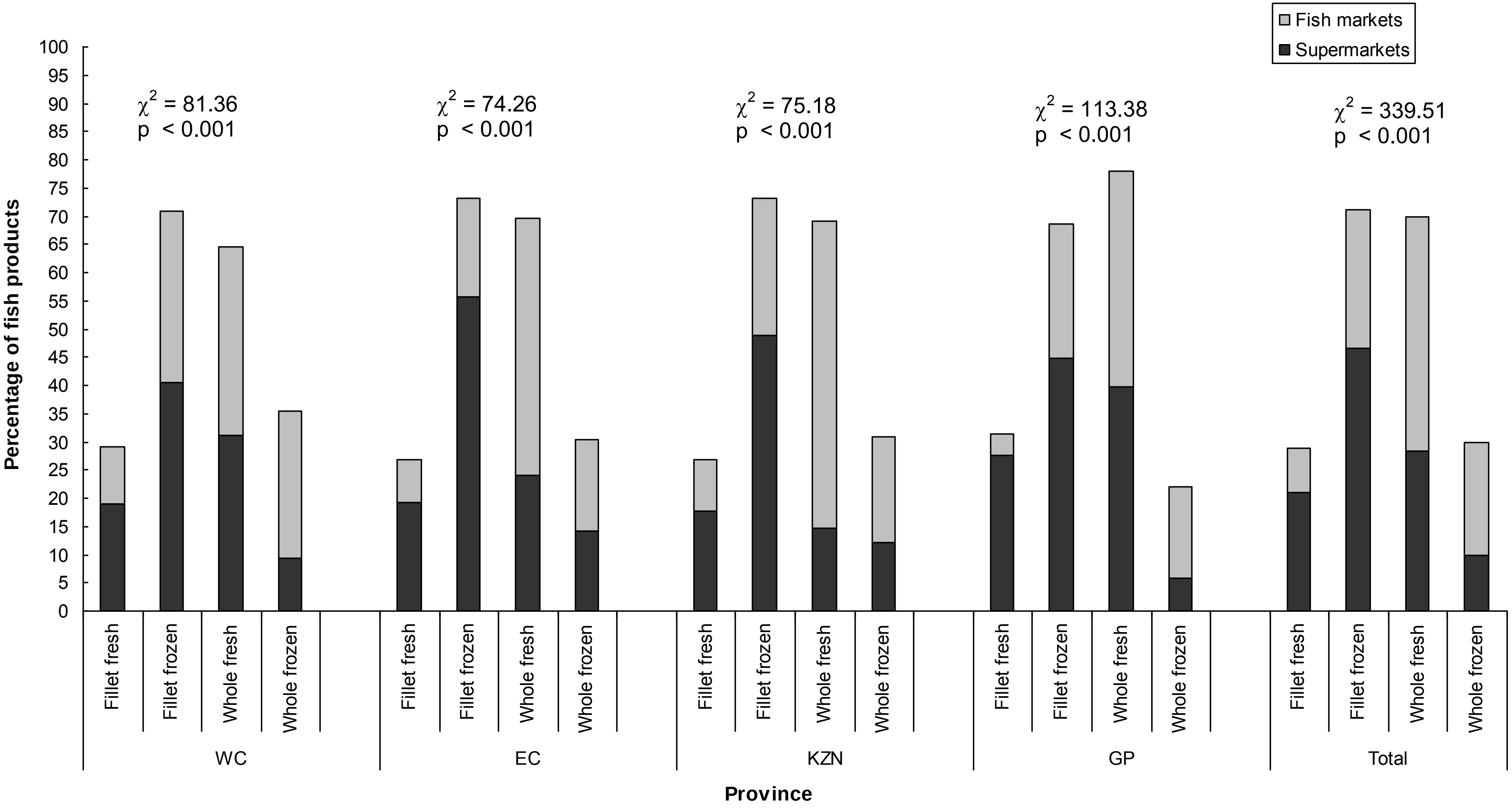


Figure 5 The relative percentages of filleted and whole fish products that were marketed in the fresh and frozen form in retail outlets.

The results of the study indicated that approximately 80% of managers in restaurants were able to provide acceptable common/market names for all their available fish which could be linked explicitly to specific species. Only about 51% of the vendors in supermarkets and 67% of the vendors in fish markets were equally well equipped to provide the aforementioned information. An estimated 17% of restaurant managers, 31% of supermarket vendors and 25% of fish market vendors were only able to identify certain fish using vague, generic group names. In some instances, group names such as 'tuna' or 'rockcod' were indicated, but the specific types/species were unknown by the interviewees. When the species could not be identified by visual inspection, the fish could not be recorded in Tables 1 and 2. Thus, the inability of interviewees to accurately identify fish species not only impairs market assessments on availability, but also impedes the already complex task faced by consumers in making sustainable fish selection. For instance, the generic name 'red fish' was frequently used at fresh fish counters to describe any red-coloured seabream (Sparidae) available in the store, which could include amongst others, Roman, slinger, santer, panga, dageraad or red stumpnose. Certain seabreams are from relatively healthy stocks, while others such as dageraad and red stumpnose are of great conservation concern.

In a number of retail outlets in GP, the vendors could only indicate that the fish they had on sale was 'red snapper'. However, when these fish were in their whole state, they were recognised on more than one occasion rather to be Mangrove red snapper/river snapper (*Lutjanus argentimaculatus*), an estuarine-dependent species which is designated for recreational fishing and is illegal to sell in South Africa (SASSI, 2010). The fact that these vendors were unable to provide vital information on the identity of these fish means that unsuspecting consumers would be unable to avoid buying illegal, locally protected fish species. Aggravating the identification problem even further is that many fish in South Africa can be referred to by more than one common name. For example, in this study, santer was regularly referred to as soldier (particularly in KZN), geelbek as Cape salmon and Cape horse mackerel as maasbanker. It is quite conceivable that such ambiguities may cause confusion to consumers, who may think that these represent different species since they are routinely sold under different names.

Surprisingly, as many as 17% of vendors in supermarkets and 8% in retail markets were unable to give any kind of interpretable common names for more than a third of the fish they were selling. For instance, the name 'white steenbras' was used synonymously with 'angelfish' to describe the same fish, 'wahoo' was used interchangeably with 'barracuda' or even 'dorado', and one meal in a KZN restaurant was referred to as 'cod' but the fine print on the menu said it was hake. In certain retail outlets in the WC and KZN, vendors were observed marketing fish as 'Roman', but inspection of the fish revealed that these specimens where actually santer seabream.

About 15% of restaurant interviewees were unsure of the origin of certain fish and around 28% were uncertain as to whether selected fish were farmed or wild. While vendors in supermarkets generally were least capable of indicating the species, origin and production method of their fish, they were the most capable of indicating which fish were the best choices in terms of sustainability and which fish were of greater conservation concern (Table 3). The vendors that were able to offer such assistance where generally those working in supermarkets that were part of the SASSI retailer participation scheme (SASSI, 2010), and they generally provided this information using the SASSI wallet card to indicate the sustainability of the species they had on sale.

Information of pre-packaged fish

In the European Union (EU), regulations have been implemented which stipulate that fresh and primary processed fish and aquaculture products may not be offered for sale unless the designated name (commercial and/or Latin names), catch area and production method (farmed or wild caught) is available to consumers at the point of sale (EC, 2000a, 2000b; 2001). South African regulations relating to the labelling of pre-packaged fish are somewhat less stringent in terms of the three aforementioned labelling criteria stipulated in the EU, requiring only the indication of a 'true description' of the 'variety' of fish and the country of origin (or production) on products (DoH, 2010; DTI, 2003).

The results of this study revealed that fish markets, in general, were non-compliant when it came to labelling their packaged frozen fish products in accordance with the applicable South African regulations. More than 87% of the frozen fish in fish markets had no labelling on the packages except for a price and, in some cases, a common name for the fish (Table 4). For more than 50% of these products, the name of the fish did not appear directly on the packaging, but rather only on the freezer compartment in which the fish were stored. Apart from the lack of adequate descriptions and country of origin labelling on these products (Table 4), the commodities were in contravention with the South African regulations (DoH, 2010; DTI, 2003) on a number of other counts not shown in the table, such as the failure to indicate the name and address of the seller and the absence of storage instruction and relevant date markings. Although it is not mandatory in South African to specify the Latin name and the production method on the labels of frozen fish, it was interesting to observe that Latin names were indicated on the labels of only 1.7% of frozen fish products in fish markets. None of the labels on frozen products in fish markets furnished information relating to the sustainability of the enclosed species or on whether the fish was farmed or wild caught.

Overall, the labelling of more than 15% of the frozen fish in supermarkets was in contravention with the South African National Regulator of Compulsory Specification (NRCS) regulations (DTI, 2003) and DoH (2010) regulations and the labelling of more than 5% of the fresh packaged fish did not fully comply with the DoH (2010) regulations. The particular shortcomings of the labelling of these products generally related to the absence of accurate descriptions of the products (including acceptable names and descriptions of the contents), the names and addresses of manufacturers or sellers, country of origin labelling, storage instructions and date markings. The labels of approximately 10% of the frozen fish products in supermarkets provided comprehensive information relating to the sustainability and conservation issues of the species; interestingly though, these all originated from one supermarket chain. Latin names were voluntarily provided on the labels of 51.7% of the frozen packaged fish and 46.6% of the fresh packaged fish in supermarkets, while the production method was indicated on 24.1% and 18.4% of these product labels, respectively.

For some packaged fish products in retail outlets where a Latin name was not provided, the designation of the common name was adequate to link the fish with a precise species. However, the provision of generic market names on certain labels made it impossible in certain instances to discriminate the precise species being sold. For example, one prominent supermarket chain in South Africa was observed selling fresh packaged fish labeled only as “dusted linefish”, which could refer to any one of more than 150 linefish caught in South Africa. Only two of these 150 linefish are considered to be optimally managed in South Africa (snoek and yellowtail), while the remainder are collapsed, threatened or overexploited (Van Schalkwyk, 2007). There is thus the possibility that the fish being sold may be of conservation concern, but the consumer would be not be aware of this if the name of the fish is not provided.

Information on fish and potential health impacts

In South Africa, escolar (*Lepidocybium flavobrunneum*) and oilfish (*Ruvettus pretiosus*) are commonly sold under the market name of ‘butterfish’ (Von der Heyden *et al*., 2010).

Table 3 Assessment of the ability of managers in restaurants and vendors in supermarkets and fish markets selling unpackaged fish to provide comprehensive information on the products being sold

	Ability to indicate:			
Rating	**Species**	**Country of origin / production**	**Farmed or wild**	**Sustainable seafood choices**
Mangers in restaurants selling fish (n = 215)				
Good	80.5%	83.7%	69.3%	13.5%
Average	16.7%	15.3%	28.4%	30.7%
Poor	2.8%	0.9%	2.3%	55.8%
Vendors at fish counters in supermarkets selling fresh fish (n = 35)				
Good	51.4%	60.0%	57.1%	31.4%
Average	31.4%	25.7%	22.9%	8.6%
Poor	17.1%	14.3%	20.0%	60.0%
Vendors at fish counters in fish markets selling fresh fish (n = 85)				
Good	67.1%	76.5%	84.7%	10.6%
Average	24.7%	23.5%	14.1%	11.8%
Poor	8.2%	0.0%	1.2%	77.6%

Rating criteria:	***Species***
Good	Able to identify all fish available by an acceptable common name
Average	Able to identify most fish by an acceptable common name, but only able to give a generic group name for certain fish (e.g. linefish, redfish or rockcod)
Poor	Unable to identify more than one third of available fish by an acceptable common name and/or misidentified certain fish
Rating criteria:	***Country of origin / production***
Good	Able to identify country of origin / production of all available fish
Average	Unsure of the country of origin / production of selected fish only
Poor	Unsure of the country of origin / production of more than half of fish or no attempt to identify origin
Rating criteria:	***Production method (wild-caught or farmed)***
Good	Able to identify all available fish as wild or farmed
Average	Able to identify most fish wild or farmed, but unsure of selected fish only
Poor	Unable to identify more than half of available fish as wild or no attempt to identify production method
Rating criteria:	***Sustainable seafood choices***
Good	Able to refer to SASSI list and assist with identifying the most sustainable choices of fish available
Average	Aware of SASSI list and able to identify some (but not all) sustainable choices
Poor	Unaware of SASSI list and could not offer assistance in making sustainable choices of fish available

Table 4 Assessment of the information available to consumers on frozen and fresh packaged fish products sold in retail outlets

	Frozen packaged fish in n = 64 fish markets					*Frozen packaged fish in n = 75 supermarkets*					*Fresh packaged fish in n = 58 supermarkets*				
		Indication on label					**Indication on label**					**Indication on label**			
Fish type (market name)	**Total products**	**Latin name**	**COOL**	**Farmed / wild**	**ND**	**Total products**	**Latin name**	**COOL**	**Farmed / wild**	**ND**	**Total products**	**Latin name**	**COOL**	**Farmed / wild**	**ND**
Angelfish	17	0.0%	5.9%	0.0%	94.1%	6	0.0%	16.7%	0.0%	83.3%	2	0.0%	50.0%	0.0%	50.0%
Barramundi						4	100.0%	100.0%	0.0%	0.0%					
Bluenose	8	0.0%	0.0%	0.0%	100.0%										
Butterfish	26	0.0%	7.7%	0.0%	92.3%	16	0.0%	31.3%	0.0%	68.8%					
Buttersnoek	6	0.0%	0.0%	0.0%	100.0%										
Cape whiting						46	100.0%	100.0%	60.9%	0.0%					
Catface rockcod	4	0.0%	0.0%	0.0%	100.0%										
Dorado	15	0.0%	13.3%	0.0%	86.7%	10	90.0%	90.0%	0.0%	10.0%					
Dory	3	0.0%	0.0%	0.0%	100.0%	2	0.0%	0.0%	0.0%	100%					
East coast sole	34	0.0%	11.8%	0.0%	88.2%	30	50.0%	53.3%	0.0%	46.7%	2	100.0%	100.0%	100.0%	0.0%
Geelbek	8	0.0%	0.0%	0.0%	100.0%										
Gurnard	8	0.0%	0.0%	0.0%	100.0%										
Haddock	12	16.7%	50.0%	0.0%	50.0%	110	50.0%	92.7%	37.3%	7.3%	3	66.6%	100.0%	0.0%	0.0%
Hake	36	5.6%	36.1%	0.0%	63.9%	146	54.8%	94.5%	31.5%	5.5%	14	85.7%	100.0%	85.7%	0.0%
Hoki hake						6	100.0%	100.0%	0.0%	0.0%					
Hottentot	5	0.0%	0.0%	0.0%	100.0%										
Jacopever	6	0.0%	0.0%	0.0%	100.0%										
Kabeljou	9	0.0%	0.0%	0.0%	100.0%										
Kingklip	36	0.0%	16.7%	0.0%	83.3%	46	86.9%	95.6%	0.0%	4.3%	16	87.5%	93.8%	0.0%	6.2%
Kippers/herring	12	0.0%	8.3%	0.0%	91.7%	52	0.0%	96.2%	0.0%	3.8%	18	0.0%	100.0%	0.0%	0.0%
Longfin tuna	6	0.0%	0.0%	0.0%	100.0%										
Maasbanker	11	0.0%	0.0%	0.0%	100.0%	6	0.0%	50.0%	0.0%	50.0%					
Mackerel	16	0.0%	0.0%	0.0%	100.0%	8	0.0%	50.0%	0.0%	50.0%					
Marlin	4	0.0%	0.0%	0.0%	100.0%										
Monk	17	0.0%	0.0%	0.0%	100.0%	4	0.0%	25.0%	0.0%	75.0%	3	0.0%	66.6%	0.0%	33.3%
Musselcracker	1	0.0%	0.0%	0.0%	100.0%	2	0.0%	0.0%	0.0%	100.0%					
Panga	8	0.0%	0.0%	0.0%	100.0%										
Portuguese sardine	7	0.0%	85.7%	0.0%	14.3%	12	2.0%	12.0%	0.0%	0.0%					
Red snapper	4	0.0%	0.0%	0.0%	100.0%										
Roman	9	0.0%	0.0%	0.0%	100.0%										
Salmon	27	11.1%	40.7%	0.0%	59.3%	74	89.2%	93.2%	71.6%	6.8%	63	46.0%	96.8%	23.8%	3.2%
Santer	7	0.0%	0.0%	0.0%	100.0%										
Silverfish	5	0.0%	0.0%	0.0%	100.0%	3	0.0%	0.0%	0.0%	100.0%					
Slinger	3	0.0%	0.0%	0.0%	100.0%										
Snoek	29	0.0%	3.4%	0.0%	96.6%	53	11.3%	58.5%	0.0%	30.2%	13	69.2%	76.9%	0.0%	23.1%
Stumpnose	5	0.0%	0.0%	0.0%	100.0%	1	0.0%	0.0%	0.0%	100.0%					
Swordfish	7	0.0%	0.0%	0.0%	100.0%										
Trout	4	0.0%	50.0%	0.0%	50.0%	6	66.7%	100.0%	66.7%	33.3%	11	36.4%	90.9%	27.3%	9.1%
West coast sole	10	0.0%	10.0%	0.0%	90.0%	1	0.0%	0.0%	0.0%	100.0%					
White steenbras	2	0.0%	0.0%	0.0%	100.0%										
Yellowbelly rockcod	2	0.0%	0.0%	0.0%	100.0%										
Yellowfin tuna	15	6.7%	26.7%	0.0%	73.3%	43	60.5%	83.7%	0.0%	16.3%	3	0.0%	66.6%	0.0%	33.3%
Yellowtail	24	0.0%	4.2%	0.0%	85.8%	4	0.0%	25.0%	0.0%	75.0%					
TOTAL	**468**	**1.7%**	**12.8%**	**0.0%**	**87.2%**	**691**	**51.7%**	**84.5%**	**24.1%**	**15.5%**	**174**	**46.6%**	**94.2%**	**18.4%**	**5.8%**

COOL = country of origin (or production) labelling
ND = no descriptors available on label, except in certain cases a common name for the fish

In fact, in this study it was found that 'butterfish' was available in more than 17% and 22% of the surveyed restaurants and retail outlets, respectively. It has long been known that both of the aforementioned fish species contain high levels of indigestible wax esters which have purgative effects, being associated with numerous outbreaks of oily diarrhoea (keriorrhea) following their consumption (Berman *et al*., 1981; Cox & Reid, 1932; Givney, 2002; Gregory, 2002). The sale and import of both of these fish has been banned in Italy, Japan and South Korea and European regulations (EC, 2003) make it mandatory to market escolar and oilfish as *L. flavobrunneum* and *R. pretiosus*, respectively. No such regulations exist in South Africa. In this study, not a single butterfish product observed in retail outlets was labelled with the Latin name, and no product carried any warning relating to the health impacts associated with its consumption in retail outlets or restaurants.

In addition, it was found that king mackerel (*Scomberomorus commerson*) was often referred to as 'couta' or 'cuda', particularly in KZN (Tables 1 and 2). King mackerel is one of the fish species listed on the FDA advisory list which should be avoided by pregnant, nursing women or young children due to its high mercury levels (FDA, 2004). However, vulnerable consumers may not realise that they are consuming this fish if it is not referred to by its generally accepted designated name, and when no Latin name is present to assist fish selection.

Conclusions

South African consumers evidently have a large variety of fish species to choose from when visiting restaurants and retail outlets. Nonetheless, in spite of an increasingly widespread understanding of the state of decline of global fisheries, the ability of local consumers to make informed purchasing decisions is probably being severely curtailed by the fact that many of the most popular fish on the domestic market are endangered (or even illegal to sell) and by the ambiguity and/or lack of information being provided on fish at the point of sale to assist with optimal choices. Disparate naming practices and low compliance with prevailing regulations is likely creating a fisheries market in South Africa that is conducive to fraud and mislabelling, signaling the need for both a revision of the adequacy of the current regulations pertaining to seafood marketing and the measures used to enforce these. The misnaming or mislabelling of fish, whether accidental or deliberate, clearly holds significant economic, health and conservation implications. Environmental groups such as SASSI continue to invest extensive efforts and resources into educating consumers on marine conservation issues and into compiling seafood lists to shift consumer purchasing decisions towards more sustainable seafood choices. However, such organisations will probably be unable to fully achieve their goals if fish species are misnamed or mislabelled in the marketplace, or if essential information on the country of origin and catch method is absent.

In the light of the results obtained here, the enhancement of transparency on the South African fisheries market will likely benefit from local regulators following the example set by the EU and stipulating the mandatory declaration on product labels of acceptable market names, species names, geographical origins and production methods of the fish being marketed. The success of such stipulations will inevitably depend on adequate enforcement including the utilisation of advanced analytical methods to verify the identity and origin of traded fish species; perhaps with larger penalties for non-compliance than those that are currently issued. Furthermore, since authorities cannot inspect or test every fish product on the market, fish suppliers in South Africa will ultimately need to take more responsibility in improving the highlighted problems existing in the current marketplace. This will likely require the sourcing and supply of more sustainable species, better compliance with government regulations and the realisation that their failure to provide vital information on fish may not only damage the marine ecosystem, but may also decrease consumer confidence in their organisation and the fishing industry as a whole.

Acknowledgements

This research on fish availability, sustainability and regulatory matters benefited from discussions with John Duncan (SASSI), Jaco Barendse (RealFish) and Pieter Truter (NRCS).

References

Anonymous (2004). Northern Cape Province: fishing and mariculture sector development strategy. [Internet document]. URL www.northern-cape.gov.za. 03 July 2010.

Bega, S. (2007). Ling's not quite the real thing. *Cape Argus*, October 27, 2007.

Berman, P., Harley, E.H. & Spark, A.A. (1981). Keriorrhoea - the passage of oil per rectum - after ingestion of marine wax esters. *South African Medical Journal*, **59**, 791-792.

Burger, J., Stern, A.H., Dixon, C., Jeitner, C., Shukla, S., Burke, S. & Gochfeld, M. (2004). Fish availability in supermarkets and fish markets in New Jersey. *Science of the Total Environment*, **333**, 89-97.

Chen, Y.C. & Chen, M.H. (2001). Heavy metal concentrations in nine species of fishes caught in coastal waters off Ann-Ping, S.W. Taiwan. *Journal of Food and Drug Analysis*, **9**, 107-114.

Costa, L. (2007). Contaminants in fish: risk-benefit considerations. *Archives of Industrial Hygiene and Toxicology*, **58**, 367-374.

Cox, W.M. & Reid, E.E. (1932). The chemical composition of oil of *Ruvettus pretiosus*, the ''castor oil fish". *Journal of the American Chemical Society*, **54**, 220-229.

DEAT (Department of Environmental Affairs and Tourism) (2005). Policy for the allocation and management of commercial fishing rights in the hake longline fishery. [Internet document]. URL www.feike.co.za/web/info/Hake%20Longline%-20Policy.doc. 14 July 2010.

DoH (Department of Health) (2010). Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act 54 of 1972), regulations relating to the labelling and advertising of foodstuffs (R. 146/2010). *South African Government Gazette*, **32975**, 3-53.

DTI (Department of Trade and Industry) (2003). Compulsory Specification for frozen fish, frozen marine molluscs and frozen products derived therefrom, VC 8017:2003, Ed. 3 (R. 979/2003). *Government Gazette*, **25172**, 3-50.

EC (European Commission) (2000a). Directive 2000/13/EC of the European Parliament and of the Council of 20 March 2000 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs. *Official Journal of the European Community*, **L109**, 29-42.

EC (European Commission) (2000b). European Council Regulation N 104/2000 of 17 December 1999 on the common organization of the markets in fishery and aquaculture products. *Official Journal of the European Community*, **L17**, 22-52.

EC (European Commission) (2001). Commission Regulation (EC) No 2065/2001 of 22 October 2001 laying down detailed rules for the application of Council Regulation (EC) No 104/2000 as regards informing consumers about fishery and aquaculture products. *Official Journal of the European Community*, **L278**, 6-8.

EC (European Communities) (2003). European Communities Labelling of Fishery and Aquaculture Products Regulations, S.I. No. 320 of 2003. [Internet document]. URL www.fsai.ie/uploadedFiles/S.I_No_320_of_2003.pdf. 19 May 2010.

FAO (Food and Agriculture Organization of the United Nations) (2008). *FAO Yearbook of Fishery and Aquaculture Statistics 2006*. Pp. 1-57. Rome, Italy: FAO.

FAO (Food and Agriculture Organization of the United Nations) (2009). *The state of world fisheries and aquaculture*. Pp. 1-153. Rome, Italy: FAO.

FDA (Food and Drug Administration) (2004). What you need to know about mercury in fish and shellfish, EPA-823-R-04-005. [Internet document]. URL www.fda.gov/Food/FoodSafety/ProductSpecificInformation/Seafood/Foodborne-PathogensContaminants/Methylmercury/default.htm. 10 July 2010.

Froese, R., & Pauly, D. (2010). FishBase. World Wide Web electronic publication. URL www.fishbase.org. 12 July 2010.

Gil, L.A. (2007). PCR-based methods for fish and fishery products authentication. *Trends in Food Science & Technology*, **18**, 558-566.

Givney, R.C. (2002). Illness associated with rudderfish/escolar in South Australia. *Communicable Disease Intelligence*, **26**, 440.

Griffiths, M.H. (2000). Long-term trends in catch and effort of commercial linefish off South Africa's Cape Province: snapshots of the 20th century. *South African Journal of Marine Science*, **22**, 81-111.

Griffiths, M.H. & Heemstra, P.C. (1995). A contribution to the taxonomy of the marine fish genus *Argyrosomus* (Perciformes: Sciaenidae), with descriptions of two new species from southern Africa. *J.L.B. Smith Institute Ichthyology Bulletin*, **65**, 40.

Gregory, J. (2002). Outbreaks of diarrhoea associated with butterfish in Victoria. *Communicable Disease Intelligence*, **26**, 439-440.

Grunert, K.G. (2002). Current issues in the understanding of consumer food choice. *Trends in Food Science & Technology*, **13**, 275-285.

Harada, M. (2005). Minamata disease: methylmercury poisoning in Japan caused by environmental pollution. *Critical Reviews in Toxicology*, **25**, 1-24.

Hites, R.A., Foran, J.A., Carpenter, D.O., Hamilton, M.C., Knuth, B.A. & Schwager, S.J. (2004). Global assessment of organic contaminants in farmed salmon. *Science*, **303**, 226-229.

Hutchings, J.A. & Reynolds, J.D. (2004). Marine fish population collapses: consequences for recovery and extinction risk. *BioScience*, **54**, 297-309.

INFOSA (2007). Fisheries in the SADC region: selected country profiles. [Internet document]. URL www.infosa.org.na/dloads/restrict/country%20profile/Fisheries-.pdf. 29 June 2010.

IUCN (International Union for Conservation of Nature) (2010). IUCN Red List of Threatened Species. Version 2010.2. [Internet document]. URL www.iucnredlist.org. 29 June 2010.

Jacquet, J.L. & Pauly, D. (2007). The rise of seafood awareness campaigns in an era of collapsing fisheries. *Marine Policy*, **31**, 308-313.

Jacquet, J.L. & Pauly, D. (2008). Trade secrets: renaming and mislabelling of seafood. *Marine Policy*, **32**, 309-318.

Logan, C.A., Alter, S.E., Haupt, A.J., Tomalty, K. & Palumbi, S.R. (2008). An impediment to consumer choice: overfished species are sold as Pacific red snapper. *Biological Conservation*, **141**, 1591-1599.

McLean, B. & Glazewski, J.I. (2009). Marine environments. In: *Fuggle & Rabie's Environmental Management in South Africa*, 2nd ed. (edited by H.A. Strydom & N.D. King). Pp. 455-511. Cape Town, South Africa: Juta.

Myers, R.A., & Worm, B. (2003). Rapid worldwide depletion of predatory fish communities. *Nature*, **423**, 280-283.

NRC (National Research Council) (2000). *Toxicological effects of methylmercury*. Pp. 147-249. Washington DC: National Academy Press.

SASSI (South African Sustainable Seafood Initiative) (2010). [Internet document]. URL www.wwfsassi.co.za. 12 July 2010.

Schlemmer, L. (1998). Gauteng: potential and challenges. In: *South Africa's Magnifying Glass: A Profile of Gauteng Province* (edited by P. Kok & R. Brandt). Pp. 23-35. Pretoria, South Africa: Human Sciences Research Council.

SeaFish (2010). Seafood export profiles: South Africa. [Internet document]. URL www.seafish.org/processors/market-insight/seafood-export-profiles. 11 May 2010.

Siebert, T. (2009). Food for thought. Sancor (South African Network for Coastal and Oceanographic Research) Newsletter, Issue 188 of Jan/Feb 2009. [Internet document]. URL http://sancor.nrf.ac.za/newsletters/past-issues/issue-188/food-for-thought. 25 June 2010.

Smith, J.L.B, & Smith, M.M. (1966). *Fishes of the Tsitsikamma Coastal National Park*. P 102. Pretoria, South Africa: National Parks Board Trust.

StatSoft, Inc. (2009). Statistica (Data Analysis Software System), version 9.0. [Internet document]. URL www.statsoft.com. 20 June 2010.

Van der Elst, R. (1997). *A Guide to Common Sea Fishes of Southern Africa*. Pp. 7-22. Cape Town, South Africa: Struik Publishers.

Van Schalkwyk, M. (2007). Media release by the minister of environmental affairs and tourism: end of commercial fishing by middle of the century unless alternatives such as aquaculture are developed. [Internet document]. URL www.mcm-deat.gov.za/press/2007/10042007.html. 14 July 2010.

Von der Heyden, S., Barendse, J., Seebregts, A.J. & Matthee, C.A. (2010). Misleading the masses: detection of mislabelled and substituted frozen fish products in South Africa. *ICES Journal of Marine Science*, **67**, 176-185.

Worm, B., Barbier, E.B. & Beaumont, N. (2006). Impacts of biodiversity loss on ocean ecosystem services. *Science*, **314**, 787-790.

CHAPTER 4

COMPARATIVE STUDY OF DIFFERENT METHODS FOR THE EXTRACTION OF DNA FROM FISH SPECIES COMMERCIALLY AVAILABLE IN SOUTH AFRICA

Abstract

Molecular methods used for the detection and identification of fish species require DNA of a high quantity and quality for successful results to be achieved. To our knowledge, no studies have been published comparing the efficiency of different methods for the extraction of DNA from fish muscle. The aim of this study was to compare five DNA extraction methods (three published methods and two commercial kits) in terms of their simplicity, reproducibility and ability to extract high yields of pure, readily amplifiable DNA from the muscle tissue of 29 fish species available in South Africa. The methods evaluated included the urea-SDS-proteinase K (MSDS), phenol-chloroform (PC) and salt extraction (SALT) methods, as well as the SureFood® PREP Allergen Kit (SF) and Wizard® Genomic DNA Purification Kit (WIZ). Considerable variations in the yields and purities of extracted DNA were observed with the different extraction methods and the individual fish species evaluated. Nonetheless, all five methods extracted DNA from the 29 fish species that was suitable for polymerase chain reaction (PCR) amplification with a cytochrome *b* (cyt *b*) gene targeting method. The SF method permitted the extraction of significantly ($P < 0.05$) higher DNA yields than all other methods evaluated, while the DNA yields obtained with the WIZ method were significantly ($P < 0.05$) the lowest. Even though DNA yields similar to those obtained with the SF method could be achieved by increasing the quantity of starting material used for the PC method, the feasibility of the PC method for routine application was limited by its labour-intensiveness and the use of hazardous reagents. Overall, the SF method might be considered the most suitable method for the extraction of high DNA yields from fish muscle tissue due to its relative safety, ease of use and applicability to high throughput extractions from multiple specimens.

Cawthorn, D.M., Steinman, H.A. & Witthuhn, R.C. (2011). Comparative study of different methods for the extraction of DNA from fish species commercially available in South Africa. *Food Control*, **22**(2), 231-244.

Introduction

In recent years, there has been an increase in the reporting of commercial fraud in the trading of fish species, emerging concurrently with the continually declining state of the world's commercial fisheries (FAO, 2009). The requirement to detect and identify fish species in foodstuffs is not only important for the prevention of adulteration, but also for the conservation of endangered species, for the protection of individuals with fish allergies and for the respect of religious and ethical beliefs (Mackie, 1996; Cėspedes *et al*., 1998; Sackesen & Adalioglu, 2003; Comi *et al*., 2005;)

DNA is reported to be the most appropriate molecule for the detection and identification of fish species in processed food products, offering numerous advantages over the analysis of proteins (Chapela *et al*., 2007). Proteins lose their biological activity shortly after the fish has died, many are heat labile, and their presence and characteristics depend on the specific cell type being analysed (Cespedes *et al.*, 1999). DNA, on the other hand, is present in all tissue types, has a greater stability at high temperatures, and the diversity afforded by the genetic code allows differentiation of closely-related species (Bartlett & Davidson, 1992; Pardo & Pérez-Villarreal, 2004).

In particular, the PCR, based on the amplification of specific DNA fragments of interest, has great potential for the detection and identification of fish, due to the fact that it is rapid, sensitive and specific (Lockley & Bardsley, 2000). PCR is often coupled with techniques such as DNA sequencing and restriction fragment length polymorphism (RFLP) to further aid in species identification (Meyer *et al*., 1995; Cespedes *et al*., 1998; Comi *et al*., 2005). The feasibility of any DNA-based technique is, however, limited by the difficulties encountered in extracting high quality DNA in sufficient quantities from complex food matrices (Yue & Orban, 2001; Wasko *et al*., 2003; Aranishi, 2006; Lopera-Barrero *et al*., 2008). DNA purity can be severely compromised by the presence of contaminants, originating either from the food matrix, such as proteins (Rijpens *et al*., 1996), lipids, phenolic compounds (Wilson, 1997) and calcium (Bickley *et al*., 1996), or from chemicals used during the DNA extraction procedure, such as phenol, sodium dodecyl sulphate (SDS) and ethylene diamine tetra-acetic acid (EDTA). Since PCR reactions may be inhibited by contaminants, it is imperative that these be removed during the DNA extraction procedure before subsequent molecular methods are applied (Marmiroli *et al*., 2003).

Traditionally, DNA extraction protocols based on the addition of organic solvents, such as phenol and chloroform, have been frequently used to isolate genomic DNA from animal species (Lopera-Barrero *et al*., 2008). Although such methods produce acceptable results for samples of diverse origins, they are time consuming and require the use of reagents that can not only chemically contaminate the extracted DNA, but which are also a health hazard (Yue & Orban, 2001). More recently, a number of commercial kits have been introduced for DNA extraction from different foods, employing either a variety of solvents and/or specialised columns containing DNA-binding substances. To date, however, no comparisons have been published comparing the efficiency of DNA extraction methods from the muscle tissue of fish, particularly those species available in South Africa. The limited reports comparing protocols for DNA extraction from fish have mainly focused on fish fins and larvae (Lopera-Barrero *et al*., 2008; Lucentini, 2006), canned tuna products (Chapela *et al*., 2007) or museum fish specimens preserved in formalin or ethanol (Chakraborty *et al*., 2006). Considering the wide range of fish products supplied by the food industry, the identification of a universal method for the extraction of DNA from fish tissue would be beneficial for a combination of molecular applications.

The aim of this study was to identify the most feasible method for the extraction of DNA from fish muscle. With this objective, the efficiency of three published methods and two commercial kits were compared for their ability to extract high yields of pure DNA suitable for PCR amplification from 29 fish species available in South Africa.

Materials and methods

Fish samples

Specimens of 26 marine and 3 freshwater fish species, comprising 25 genera in 19 families of ray-finned fishes (*Actinopterygii*) (Table 1) were obtained from major commercial trawling companies, fish processing facilities, the South African Department of Agriculture, Forestry and Fisheries (DAFF), as well as the Aquaculture Division, Stellenbosch University, South Africa. All specimens were morphologically identified by fish taxonomists and were stored at -20 °C prior to DNA extraction.

Tissue preparation

Samples of muscle tissue were excised from the lateral muscle (skin removed) on the right-hand side of the fish specimens and were minced with sterile razor blades prior to DNA extraction.

DNA extractions

Genomic DNA was extracted from all 29 fish species using five different extraction methods. All DNA extractions were conducted in triplicate assays for each fish sample.

Modified urea-SDS-proteinase K method

DNA was extracted from all fish specimens according to the modified urea-SDS-proteinase K (MSDS) method (Aranishi, 2005), described for the extraction of DNA from mackerel species. Minced fish muscle samples (50 mg) were transferred to 2 ml sterile microcentrifuge tubes, followed by the addition of 500 µl TESU6 buffer (10 mM Tris-HCl, pH 8.0; 20 mM EDTA, pH 8.0; 2% (m/v) SDS; 6 M urea) (all supplied by Sigma-Aldrich, Gauteng, South Africa) and 20 µl of 10 $mg.ml^{-1}$ proteinase K (Sigma-Aldrich). The content of the tubes was mixed using a vortex, followed by incubation at 55 °C for 15 min. Thereafter, a 0.1 volume of 5 M NaCl (Sigma-Aldrich) was added and mixed by inversion. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich) was added and mixed by inversion. Samples were centrifuged (Eppendorf Centrifuge 5415D) at 10 000 g for 5 min and the upper aqueous phase was collected in a new sterile microcentrifuge tube. DNA was precipitated with a 0.6 volume of isopropanol (Sigma-Adrich), washed with 70% (v/v) ethanol (Sigma-Aldrich), air dried and resuspended in 100 µl 10T0.1E buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0) (Sigma-Aldrich). The extracted DNA was stored at -20 °C until further use.

Modified phenol-chloroform method

A modified phenol-chloroform (PC) method, previously described for the extraction of DNA from the muscle tissue of tilapia species (Bardakci & Skibinski, 1994), was utilised for the extraction of DNA from all fish specimens. Minced fish muscle samples (50 mg) were transferred to 2 ml sterile microcentrifuge tubes, followed by the addition of 500 µl STE buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8; 0.1 M NaC1) (Sigma-Aldrich), 15 µl 20% (m/v) SDS (Sigma-Aldrich) and 30 µl of 10 $mg.ml^{-1}$ proteinase K (Sigma-Aldrich). Samples were mixed briefly using a vortex, incubated at 50 °C for 1 h and then centrifuged at 10 000 g for 5 min. DNA was purified using successive extractions with 250 µl pure phenol (Sigma-Aldrich), phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich) and chloroform:isoamyl alcohol (24:1) (Sigma-Aldrich), respectively. DNA was precipitated with 750 µl ice-cold absolute ethanol (Sigma-Aldrich). Following centrifugation (10 000 g, 10 min), the DNA pellet was washed with 70% (v/v) ethanol (Sigma-Aldrich), air dried and resupended in 100 µl 10T0.1E buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0) (Sigma-Aldrich). The extracted DNA was stored at -20 °C until further use.

Salt extraction method

DNA was extracted from all fish specimens according to the salt extraction (SALT) method, utilised previously for the extraction of DNA from shrimp muscle (Aljanabi & Martinez, 1997) and modified for the extraction of DNA from fish caudal fins and larvae (Lopera-Barrero *et al.*, 2008). Minced fish muscle samples (50 mg) were immersed in 400 µl of lysis buffer (10 mM Tris-HCl, pH 8.0; 2 mM EDTA pH 8.0; 0.4 M NaCl) (Sigma-Aldrich), 40 µl of 20% (m/v) SDS (Sigma-Aldrich) and 20 µl of 10 $mg.ml^{-1}$ proteinase K (Sigma-Aldrich) and were mixed by vortexing. Following incubation at 65 °C for 1 h, 300 µl of 6 M NaCl (Sigma-Aldrich) was added to each sample. Samples were vortexed at maximum speed for 30 s and then centrifuged at 10 000 g for 30 min. The upper aqueous phase from each sample was collected in a new sterile microcentrifuge tube and an equal volume of isopropanol (Sigma-Aldrich) was added to each sample and mixed by vortexing. Samples were incubated at -20 °C for 1 h and were then centrifuged at 10 000 g for 20 min. The DNA pellet was washed with 70% (v/v) ethanol (Sigma Aldrich), air dried and resupended in 100 µl 10T0.1E buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0) (Sigma-Aldrich). The extracted DNA was stored at -20 °C until further use.

SureFood® PREP Allergen kit

The SureFood® PREP allergen kit (SF) (r-Biopharm, supplied by AEC-Amersham, Cape Town, South Africa) was utilised for the extraction of DNA from the muscle of the fish species, following the instructions of the test kit manufacturer. As recommended for DNA extraction from fish tissue, 500 mg samples of minced fish muscle were transferred to sterile microcentrifuge tubes, followed by the addition of 1 ml of lysis buffer (r-Biopharm) and 40 µl of 10 $mg.ml^{-1}$ proteinase K (r-Biopharm). The samples were vortexed briefly and incubated at 65 °C for 1 h. Sample lysates were centrifuged (13 000 g, 2 min) and the liquid supernatant was transferred to a new microcentrifuge tube. Following centrifugation (13 000 g for 2 min), 650 µl of the supernatant was transferred directly into a spin filter (r-Biopharm) placed in a 2 ml receiver tube. The receiver tube containing the spin filter was centrifuged (13 400 g for 1 min) and the spin filter was discarded. A volume of 500 µl of binding buffer (r-Biopharm) was added to the filtrate and vortexed thoroughly. From this solution, 650 µl was transferred directly to a new spin filter placed in a fresh receiver tube, incubated at room temperature for 1 min, and then centrifuged at 13 400 g for 2 min. The same spin filter was placed into a new receiver tube and the remaining 650 µl of solution was added and incubated at room temperature for 1 min. The receiver tube containing the spin filter was centrifuged at 13 400 g for 2 min. After removing the filtrate from the receiver tube with a pipette, the spin filter was returned to the receiver tube, and 550 µl of pre-wash buffer (r-Biopharm) was added to the spin filter. The receiver tube containing the spin filter was centrifuged at 13 400 g for 1 min, the filtrate was once again removed, and the spin filter was returned to the receiver tube. The same wash and centrifugation step was repeated twice with 550 µl of wash buffer, the filtrate removed and the spin filter returned to the receiver tube. The spin filter was dried by centrifugation at 13 400 g for 2 min. The spin filter was transferred to a new receiver tube, 100 µl of pre-heated (65 °C) elution buffer (r-biopharm) was added and this was incubated at 65 °C for 3 min. DNA was eluted from the spin filter by centrifugation (10 000 rpm for 2 min) and was stored at -20 °C until further use.

2.3.5. Wizard® Genomic DNA Purification Kit (WIZ)

DNA was extracted from all fish specimens following the instructions of the manufacturer of the Wizard® Genomic DNA Purification Kit (WIZ) (Promega, supplied by Whitehead Scientific, Cape Town, South Africa), specifically following the protocol described for the isolation of DNA from animal tissue. Samples of 20 mg of minced fish muscle were transferred to microcentrifuge tubes containing 600 µl of chilled nuclei lysis solution (Promega). Samples were homogenised for 10 s and were incubated at 65 °C for 30 min. After allowing the lysate to cool to room temperature, 3 µl of RNase solution (Promega) was added and mixed by inversion. Samples were incubated at 37 °C for 30 min and then allowed to cool for 5 min. A volume of 200 µl of protein precipitation solution (Promega) was added to the tubes, which were vortexed at maximum speed for 30 s and then chilled on ice for 5 min. Samples were centrifuged at 15 000 g for 3 min. The supernatants were transferred to new microcentrifuge tubes containing 600 µl of room temperature isopropanol (Sigma Aldrich) and mixed by inversion until white thread-like DNA strands were visible. DNA was then pelleted by centrifugation at 15 000 g for 1 min. After removal of the liquid supernatant, the DNA pellet was washed with 600 µl of 70% (v/v) ethanol (Sigma-Aldrich), followed by centrifugation at 15 000 g for 1 min. Residual ethanol was aspirated and samples were air dried. DNA was rehydrated overnight by incubation with 100 µl of DNA rehydration solution (Promega) at 4 °C before storage at -20 °C.

DNA concentration, yield and purity

Known volumes of DNA extracts were diluted to 2 ml in double distilled water and aliquots of the diluted DNA were transferred to separate quartz cuvettes. The diluted DNA solutions were quantified and assessed for impurities by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) in a spectrophotometer (Beckman Coulter DU530, Beckman Instruments, Fullerton, USA). DNA concentrations were calculated by multiplying the A_{260} measurement by the dilution factor and then by 50, based on the relationship that an A_{260} of 1.0 equals 50 µg.ml^{-1} pure DNA (Sambrook & Russell, 2001). DNA yields were calculated by multiplying the DNA concentration value by the final volume of DNA extracted with each method. DNA purities were determined by calculating the A_{260} / A_{280} ratios. Samples calculated to have A_{260} / A_{280} ratios of approximately 1.7 - 2.1 were assumed to be pure samples, free from protein and/or RNA contamination (Aljanabi & Martinez, 1997; Rapley, 2000; Wasko *et al*., 2003; Ferrara *et al*., 2006; Lopera-Barrero *et al*., 2008).

Polymerase chain reaction

The oligonucleotide primers Cyt bL (5´- CCA TCC AAC ATC TCA GCA TGA TGA AA-3´) and Cyt bH (5´-CCC CTC AGA ATG ATA TTT GTC CTC A-3´) were utilised to amplify a 359 base pair (bp) fragment of a conserved region of the cytochrome *b* (cyt *b*) gene (Bartlett & Davidson, 1992). The PCR reaction mixture (50 µl total volume) contained 10 mM Tris-HCl, pH 8.0; 50 mM KCl; 0.2 µM of each cyt *b* primer (Operon, supplied by Southern Cross Biotechnologies, Cape Town, South Africa), 1.25 U *Taq* DNA polymerase (Super-Therm, supplied by Southern Cross Biotechnologies), 2 mM $MgCl_2$ (Super-Therm), 0.2 mM of each dNTP (AB gene, supplied by Southern Cross Biotechnologies) and 2 - 3 µl (*ca*. 90 ng - 9.0 µg) DNA template.

PCR was carried out in a Mastercycler Personal (Eppendorf, Germany) using the following thermal cycling conditions: initial denaturation at 95 °C for 5 min; followed by 30 cycles of denaturation at 94 °C for 30 s; primer annealing at 55 °C for 30 s; and chain elongation at 72 °C for 1 min. Final elongation was performed at 72 °C for 7 min. PCR products (5 µl) were separated on a 1.5% (m/v) agarose (Sigma-Adrich) gel, containing 0.02 µl.ml^{-1} ethidium bromide, in 0.5 x TBE electrophoresis buffer. The separated PCR fragments were visualised under an ultraviolet transilluminator (Vilber Lourmat, France).

Limit of detection

The limit of detection (LOD) of the cyt *b* PCR was compared utilising the DNA extracted with each method by preparing 10-fold serial dilutions of the extracts and performing PCR amplification on these diluted extracts as previously described. The LOD for each method was assigned at the lowest concentration of DNA that produced a visible PCR product of the expected size on an agarose gel. Each diluted extract was PCR amplified at least in duplicate.

Standardisation of DNA extraction methods

In order to directly compare the efficiency of the DNA extraction methods, the amount of starting material used for extraction was standardised by repeating the SureFood® PREP Allergen protocol and the Wizard® Genomic DNA Purification protocol as previously described, using 50 mg fish muscle tissue as starting material. Thereafter, the phenol-chloroform method was repeated as described with 500 mg fish muscle tissue as starting material, for direct comparison with the results obtained with the SureFood® PREP Allergen kit. The quality and quantity of the DNA extracted with the standardised protocols was assessed spectrophotometrically.

Statistical analysis

All statistical analyses were performed using Statistica™ 7.1 (StatSoft, Inc., 2006). For comparison of the concentrations of DNA extracted from all fish species with the different DNA extraction methods, a two-way cross-classification analysis of variance (ANOVA) was performed. A two-way ANOVA was also utilised to compare the purities of the DNA extracted from all fish species with the different DNA extraction methods. Differences were considered statistically significant at a level of 5% ($P < 0.05$). A Bonferroni multiple comparisons procedure was used when interactions were significant in order to interpret which interaction effects differed. The differences in the concentrations and purities of the DNA extracted with the different methods were also compared using the Bootstrap multiple comparisons procedure, excluding the variations existing between the individual fish species, and taking all the fish as independent replicates.

Results and discussion

DNA extraction methods

In this study, five different DNA extraction methods were compared in terms of their ability to extract high yields of pure DNA from the muscle tissue of a wide range of fish species available in South Africa (Table 1). The MSDS, PC, SALT, SF and WIZ methods employ different principles of separation, as illustrated in Table 2.

Quantitative and qualitative analysis of DNA extracts

The concentrations, yields and purities of the DNA extracted from all 29 fish species with the five different DNA extraction methods, as determined using absorbance values at 260 nm (A_{260}) and 280 nm (A_{280}), are presented in Table 1. Spectrophotometric evaluations of the quality and quantity of DNA using A_{260} and A_{280} values have been employed by various researchers to compare different DNA extraction methods (Peano *et al*., 2004; Kakihara *et al*., 2006; Ren *et al*., 2006; Chapela *et al.*, 2007; Di Pinto *et al*., 2007; Mafra *et al*., 2008).

DNA concentrations and yields with methods carried out according to protocols

Table 1 shows the wide range of DNA yields obtained from the different fish using the five different DNA extraction methods when these were performed according to the protocols. Statistical analysis, employed to identify where the DNA yields and purities differed significantly, showed that significant interactions existed between the different extraction methods and between the individual fish species. A graphical representation of the 9730 interactions between all the fish species and all the extraction methods on one plot made for very complex interpretation, thus the interactions were condensed individually for each fish species (Fig. 1) and for each extraction method (Fig. 2).

From the data in Table 1, it is clear that when the extraction methods were performed according to the protocols, the SF method consistently extracted the highest yields of DNA from all fish species. This could be attributed to the fact that this method required a greater amount of starting material (500 mg fish muscle) for DNA extraction than that required by the MSDS, PC and SALT methods (50 mg fish muscle) and the WIZ method (20 mg fish muscle). The higher DNA yields obtained with the SF method were significant ($P < 0.05$) for 26 of the 29 fish species (90%), but were not significant ($P > 0.05$) in the case of the carpenter seabream, white stumpnose and east coast sole (Fig. 1).

Table 1 Comparison of concentrations, yields and purities of DNA extracted from 29 fish species with five extraction methods

Order	Family	Species name	Common name	Method	DNA concentration range (ng.ul^{-1})	DNA yield range (ug)	A_{260}/A_{280} range	PCR	PCR LOD (pg.ul^{-1})
Perciformes	Scombridae	*Thunnus albacares*	Yellowfin tuna	MSDS	96.0 - 132.0	9.6 - 13.2	1.4 - 1.8	+	0.12
				PC	151.2 -189.6	15.2 - 18.96	1.3 - 2.0	+	0.17
				SALT	69.6 - 108.0	7.0 - 10.8	2.0 - 3.2	+	0.20
				SF	662.4 - 907.2	66.2 - 90.7	2.1 - 2.3	+	0.08
				WIZ	19.2 - 45.6	1.9 - 4.6	1.9 - 2.0	+	0.23
				PC_{500}	1956.0 - 2160.0	195.6 - 216.0	1.9 - 2.6	+	ND
				WIZ_{50}	21.6 - 36.0	2.2 - 3.6	1.5 - 1.9	+	ND
		Thunnus alalunga	Longfin tuna (albacore)	MSDS	55.2 - 72.0	5.5 - 7.2	1.4 - 1.9	+	0.58
				PC	175.2 - 220.8	17.5 - 22.1	1.6 - 1.7	+	0.20
				SALT	84.0 - 134.4	8.4 - 13.4	1.3 - 4.0	+	0.20
				SF	1195.2 - 1296.0	119.5 - 129.6	2.1 - 2.5	+	0.10
				WIZ	28.8 - 67.2	2.9 - 6.7	1.8 - 2.0	+	0.53
				PC_{500}	1344.0 - 1632.0	134.4 - 163.2	1.8 - 2.1	+	ND
				WIZ_{50}	28.8 - 57.6	2.9 - 5.8	1.4 - 2.0	+	ND
	Sciaenidae	*Atractoscion aequidens*	Cape salmon (geelbek)	MSDS	160.8 - 216.0	16.1 - 21.6	1.5 - 1.7	+	0.16
				PC	244.8 - 288.0	24.5 - 28.8	1.7 - 2.0	+	0.26
				SALT	84.0 - 144.0	84.0 - 14.4	1.4 - 4.2	+	0.12
				SF	1339.2 - 1497.6	133.9 - 149.8	1.9 - 2.2	+	0.10
				WIZ	24.0 - 50.4	2.4 - 5.0	1.6 - 1.7	+	0.38
				PC_{500}	2726.4 - 3307.2	272.6 - 330.7	1.8 - 2.0	+	ND
				WIZ_{50}	14.4 - 38.4	1.4 - 3.8	1.8 - 2.0	+	ND
		Argyrosomus inodorus	Silver kob (mild meagre)	MSDS	72.0 - 98.4	7.2 - 9.5	1.4 - 1.5	+	0.94
				PC	453.6 - 532.8	45.4 - 53.3	2.5 - 3.8	+	0.51
				SALT	2.4 - 62.4	0.2 - 6.2	1.2 - 4.3	+	0.38
				SF	768.0 - 892.8	76.8 - 89.3	2.1 - 2.5	+	0.08
				WIZ	14.4 - 31.2	1.4 - 3.1	1.6 - 2.0	+	0.24
				PC_{500}	2640.0 - 3256.8	264.0 - 325.7	1.9 - 2.3	+	ND
				WIZ_{50}	12.0 - 31.2	1.2 - 3.1	1.6 - 2.5	+	ND
		Argyrosomus japonicus	Dusky kob (Japanese meagre)	MSDS	62.4 - 93.6	6.2 - 9.4	1.5 - 1.7	+	0.82
				PC	192.0 - 259.2	19.2 - 25.9	1.8 - 2.7	+	0.23
				SALT	141.6 - 182.4	14.2 - 18.2	2.1 - 2.3	+	0.25
				SF	1440.0 - 1728.0	144.0 - 172.8	1.9 - 2.0	+	0.02
				WIZ	26.4 - 55.2	2.6 - 5.5	1.6 - 1.9	+	0.36
				PC_{500}	2064.0 - 2664.0	206.4 - 266.4	1.9 - 2.6	+	ND
				WIZ_{50}	28.8 - 38.4	2.9 - 3.8	1.5 - 1.7	+	ND
	Sparidae	*Argyrozona argyrozona*	Carpenter seabream	MSDS	151.2 - 206.4	15.1 - 20.6	1.4 - 1.5	+	0.17
				PC	237.6 - 336.0	23.8 - 33.6	1.8 - 4.2	+	0.30
				SALT	144.0 - 206.4	14.4 – 20.6	1.6 - 3.8	+	0.20
				SF	336.0 - 590.4	33.6 - 59.0	2.3 - 2.6	+	0.05
				WIZ	4.80 - 52.80	0.5 - 5.3	1.6 - 2.0	+	0.30
				PC_{500}	1440.0 - 1800.0	144.0 - 180.0	2.0 - 2.3	+	ND
				WIZ_{50}	4.8 - 40.8	0.5 - 4.1	1.3 - 2.0	+	ND
		Chrysoblephus laticeps	Roman seabream	MSDS	141.6 - 177.6	14.6 - 17.8	1.3 - 1.5	+	0.16
				PC	177.6 - 240.0	17.6 - 24.0	1.2 - 1.7	+	0.19
				SALT	24.0 -86.4	2.4 - 8.6	1.6 - 1.9	+	0.55
				SF	417.6 - 571.2	41.8 - 57.1	2.2 - 2.6	+	0.05
				WIZ	2.4 - 28.8	0.2 - 2.9	1.0 - 2.0	+	0.22
				PC_{500}	1032.0 - 1440.0	103.2 – 144.0	1.4 - 1.8	+	ND
				WIZ_{50}	9.6 - 26.4	1.0 - 2.6	1.5 - 2.0	+	ND
		Cheimerius nufar	Santer seabream	MSDS	72.0 - 144.0	7.2 - 14.4	1.4 - 1.6	+	0.19
				PC	244.8 - 374.4	24.5 - 37.4	2.5 - 3.4	+	0.34

Table 1 (continued)

Order	Family	Species name	Common name	Method	DNA concentration range (ng.ul^{-1})	DNA yield range (ug)	A_{260}/A_{280} range	PCR	PCR LOD (pg.ul^{-1})
Perciformes	Sparidae	*Cheimerius nufar*	Santer seabream	SALT	384.0 - 480.0	38.4 – 48.0	3.6 - 4.4	+	0.50
				SF	940.8 - 1248.0	94.1 - 124.8	2.0 - 2.2	+	0.02
				WIZ	19.2 - 50.4	1.9 - 5.0	1.8 - 2.0	+	0.34
				PC_{500}	2304.0 - 2880.0	230.4 - 288.0	1.9 - 2.6	+	ND
				WIZ_{50}	16.8 - 45.6	1.7 - 4.6	1.4 - 1.8	+	ND
		Rhabdosargus globiceps	White stumpnose	MSDS	48.0 - 129.6	4.8 - 13.0	1.0 - 1.7	+	0.74
				PC	144.0 - 288.0	14.4 - 28.8	1.7 - 2.0	+	0.22
				SALT	69.6 - 206.4	7.0 - 20.6	2.9 - 4.5	+	0.16
				SF	201.0 - 393.6	20.1 - 39.4	2.1 - 2.2	+	0.03
				WIZ	19.2 - 45.6	1.9 - 4.6	1.6 - 1.8	+	0.34
				PC_{500}	1272.0 - 1680.0	127.2 - 168.0	2.0 - 2.6	+	ND
				WIZ_{50}	16.8 - 38.4	1.7 - 3.8	1.6 - 1.8	+	ND
	Cichlidae	*Oreochromis mossambicus*	Mozambique tilapia	MSDS	48.0 - 110.4	4.8 - 11.0	1.4 - 1.7	+	0.11
				PC	237.6 - 297.6	23.8 - 29.8	1.7 - 1.9	+	0.28
				SALT	86.40 - 134.40	8.6 - 13.4	3.9 - 4.8	+	0.11
				SF	1022.4 - 1296.0	102.2 - 129.6	1.7 - 2.4	+	0.01
				WIZ	12.0 - 45.6	1.2 - 4.6	1.7 - 2.0	+	0.29
				PC_{500}	1644.0 - 1788.0	164.4 - 178.8	1.9 - 2.3	+	ND
				WIZ_{50}	21.6 - 48.0	2.2 - 4.8	1.5 - 1.8	+	ND
	Gempylidae	*Thyrsites atun*	Snoek	MSDS	48.0 - 72.0	4.8 - 7.2	1.5 - 2.0	+	0.53
				PC	237.6 - 283.2	23.8 - 28.3	1.8 - 1.9	+	0.26
				SALT	213.6 - 240.0	21.4 - 24.0	2.5 - 4.8	+	0.23
				SF	1440.0 - 1852.8	144.0 - 185.3	2.1 - 2.3	+	0.02
				WIZ	21.6 - 28.8	2.2 - 2.9	1.7 - 2.2	+	0.26
				PC_{500}	989.5 - 1495.2	99.0 - 149.5	1.6 - 2.3	+	ND
				WIZ_{50}	16.8 - 45.6	1.7 - 4.6	1.4 - 1.8	+	ND
	Bramidae	*Brama brama*	Angelfish (Atlantic pomfret)	MSDS	156.0 - 204.0	15.6 - 20.4	1.8 - 1.9	+	0.18
				PC	300.0 - 321.6	30.0 - 32.2	1.7 - 1.9	+	0.31
				SALT	93.6 - 170.4	9.4 - 17.0	1.9 - 3.5	+	0.13
				SF	1574.4 - 1780.8	157.4 - 178.1	2.2 - 2.5	+	0.02
				WIZ	28.8 - 48.0	2.9 - 4.8	1.8 - 2.0	+	0.36
				PC_{500}	1845.6 - 1968.0	184.6 - 196.8	1.9 - 2.1	+	ND
				WIZ_{50}	28.8 - 48.0	2.9 - 4.8	1.7 - 2.0	+	ND
	Trichiuridae	*Lepidopus caudatus*	Ribbon snoek (silver scabbardfish)	MSDS	96.0 - 141.6	9.6 - 14.2	1.4 - 1.6	+	0.11
				PC	211.2 - 244.8	21.1 - 24.8	1.7 – 2.0	+	0.22
				SALT	48.0 - 240.0	4.8 - 24.0	2.0 - 5.0	+	0.12
				SF	542.4 - 748.8	54.2 - 74.9	2.0 - 2.5	+	0.06
				WIZ	19.2 - 57.6	1.9 - 5.8	1.7 - 2.0	+	0.45
				PC_{500}	1567.2 - 1680.0	156.7 - 168.0	1.9 - 2.3	+	ND
				WIZ_{50}	19.2 - 55.2	1.9 - 5.5	1.6 - 1.8	+	ND
	Carangidae	*Seriola lalandi*	Yellowtail (yellowtail amberjack)	MSDS	110.4 - 192.0	11.0 - 19.2	1.6 - 1.8	+	0.16
				PC	283.2 -309.6	28.3 - 31.0	1.8 - 1.9	+	0.29
				SALT	55.2 - 72.0	5.5 - 7.2	2.0 - 2.6	+	0.65
				SF	844.8 - 1104.0	84.5 - 110.4	1.8 – 2.0	+	0.01
				WIZ	14.4 - 33.6	1.4 - 3.4	1.8 - 2.0	+	0.26
				PC_{500}	1656.0 - 2304.0	165.6 - 230.4	2.1 - 2.8	+	ND
				WIZ_{50}	7.2 - 26.4	0.7 - 2.6	1.8 - 2.0	+	ND
		Trachurus capensis	Cape horse mackerel	MSDS	175.2 - 216.0	17.5 - 21.6	1.7 - 1.9	+	1.92
				PC	292.8 - 336.0	29.3 - 33.6	2.3 - 4.4	+	0.32
				SALT	182.4 - 230.4	18.2 - 23.0	2.5 - 2.8	+	0.20
				SF	1339.2 - 1440.0	133.9 - 144.0	2.1 - 2.3	+	0.01
				WIZ	21.6 - 50.4	2.2 - 5.0	1.8 - 1.9	+	0.36

Table 1 (continued)

Order	Family	Species name	Common name	Method	DNA concentration range (ng.ul^{-1})	DNA yield range (ug)	A_{260}/A_{280} range	PCR	PCR LOD (pg.ul^{-1})
				PC_{500}	960.0 - 1920.0	96.0 - 192.0	1.9 - 2.6	+	ND
				WIZ_{50}	19.2 - 45.6	1.9 - 4.6	1.6 - 1.8	+	ND
Perciformes	Coryphaenidae	*Coryphaena hippurus*	Dorado (common dolphinfish)	MSDS	168.0 - 240.0	16.8 - 24.0	1.6 - 1.9	+	0.20
				PC	384.0 - 446.4	38.4 - 44.6	1.8 – 3.5	+	0.42
				SALT	168.0 - 432.0	16.8 - 43.2	1.8 - 3.9	+	0.38
				SF	1814.4 - 1920.0	90.7 - 96.0	2.0 - 2.1	+	0.02
				WIZ	31.2 - 48.0	3.1 - 4.8	1.6 – 2.0	+	0.42
				PC_{500}	2952.0 - 3360.0	295.2 - 336.0	2.0 - 2.8	+	ND
				WIZ_{50}	31.2 - 40.8	3.1 - 4.1	1.6 - 1.9	+	ND
Salmoniformes	Salmonidae	*Salmo salar*	Atlantic salmon	MSDS	518.4 - 705.6	51.8 - 70.6	1.4 - 1.5	+	0.60
				PC	333.6 - 705.6	51.8 - 70.6	2.0 - 3.5	+	0.50
				SALT	643.2 - 696.0	64.3 - 69.6	2.6 - 3.8	+	0.68
				SF	1123.2 - 1248.0	112.3 - 124.8	2.2 - 2.4	+	0.01
				WIZ	24.0 - 45.6	2.4 - 4.6	1.9 - 2.0	+	0.32
				PC_{500}	2472.0 - 3120.0	247.2 - 312.0	1.9 - 2.5	+	ND
				WIZ_{50}	21.6 - 36.0	2.2 - 3.6	1.8 - 1.9	+	ND
		Oncorhynchus keta	Chum salmon	MSDS	417.6 - 487.2	41.8 - 48.7	1.6 - 2.8	+	0.44
				PC	285.6 - 331.2	28.6 - 33.1	1.5 - 1.6	+	0.30
				SALT	216.0 - 283.2	21.6 - 28.3	2.0 - 2.5	+	0.26
				SF	720.0 - 960.0	72.0 - 96.0	2.0 - 2.1	+	0.01
				WIZ	19.2 - 50.4	1.9 - 5.0	1.6 - 2.0	+	0.41
				PC_{500}	1860.0 - 2054.4	186.0 - 205.4	1.6 - 2.0	+	ND
				WIZ_{50}	9.6 - 40.8	1.0 - 4.1	1.7 - 2.0	+	ND
		Oncorhynchus mykiss	Rainbow trout	MSDS	268.8 - 324.0	26.8 - 32.4	1.4 - 1.6	+	0.29
				PC	151.2 - 192.0	15.1 - 19.2	1.6 - 1.8	+	0.18
				SALT	326.4 - 405.6	32.6 - 40.6	1.9 - 2.1	+	0.36
				SF	1387.2 - 1632.0	138.7 - 163.2	2.1 - 2.3	+	0.01
				WIZ	28.8 - 48.0	2.9 - 4.8	1.7 - 1.8	+	0.36
				PC_{500}	964.0 - 960.0	96.4 - 96.0	1.8 - 2.0	+	ND
				WIZ_{50}	21.6 - 48.0	2.2 - 4.8	1.6 - 1.8	+	ND
Zeiformes	Zeidae	*Zeus capensis*	Cape dory	MSDS	196.8 - 252.0	19.7 - 25.2	1.3 - 1.5	+	0.24
				PC	364.8 - 410.4	36.5 - 41.0	1.7 - 1.9	+	0.38
				SALT	187.2 - 240.0	18.7 - 24.0	2.3 - 3.3	+	0.22
				SF	2016.0 - 2342.4	201.6 - 234.2	1.9 - 2.4	+	0.02
				WIZ	55.2 - 88.8	5.5 - 8.9	1.7 - 1.8	+	0.72
				PC_{500}	2472.0 - 3360.0	247.2 - 336.0	1.8 - 2.4	+	ND
				WIZ_{50}	50.4 - 72.0	5.0 - 7.2	1.7 - 1.8	+	ND
Lophiiformes	Lophiidae	*Lophius vomerinus*	Cape monk (devil anglerfish)	MSDS	36.0 - 96.0	3.6 - 9.6	1.3 - 1.9	+	0.62
				PC	261.6 - 384.0	26.2 - 38.4	1.8 - 3.7	+	0.34
				SALT	36.0 - 72.0	3.6 - 7.2	1.3 - 2.0	+	0.58
				SF	912.0 - 1228.8	91.2 - 122.9	2.0 - 2.5	+	0.01
				WIZ	14.4 - 57.6	1.4 - 5.8	1.6 - 2.0	+	0.41
				PC_{500}	1488.0 - 1968.0	148.8 - 196.8	2.0 - 2.6	+	ND
				WIZ_{50}	24.0 - 48.0	2.4 - 4.8	1.7 - 2.0	+	ND
Ophidiiformes	Ophidiidae	*Genypterus capensis*	Kingklip	MSDS	96.0 - 165.6	9.6 - 16.6	1.7 - 2.0	+	0.13
				PC	182.4 - 220.8	18.2 - 22.1	1.6 - 1.9	+	0.21
				SALT	156.0 - 199.2	15.6 - 19.9	1.4 - 2.3	+	0.19
				SF	432.0 - 691.2	43.2 - 69.1	2.2 - 2.3	+	0.06
				WIZ	14.4 - 50.4	1.4 - 50.4	1.8 – 2.0	+	3.84
				PC_{500}	1680.0 - 1944.0	168.0 - 194.4	1.7 - 1.9	+	ND
				WIZ_{50}	26.4 - 38.4	2.6 - 3.8	1.6 - 1.8	+	ND

Table 1 (continued)

Order	Family	Species name	Common name	Method	DNA concentration range (ng.ul^{-1})	DNA yield range (ug)	A_{260}/A_{280} range	PCR	PCR LOD (pg.ul^{-1})
Gadiformes	Merlucciidae	*Merluccius capensis*	Shallow-water Cape hake	MSDS	67.2 - 134.4	6.7 - 13.4	1.3 - 1.8	+	0.10
				PC	477.6 - 511.2	47.8 - 51.1	2.2 - 3.5	+	0.49
				SALT	552.0 - 720.0	55.2 - 72.0	3.1 - 3.8	+	0.65
				SF	3648.0 - 4272.0	364.8 - 427.2	1.9 - 2.8	+	0.04
				WIZ	38.4 - 62.4	3.8 - 6.2	1.7 - 2.3	+	0.53
				PC_{500}	3960.0 - 4344.0	396.0 - 434.4	2.0 - 2.6	+	ND
				WIZ_{50}	19.2 - 38.4	1.9 - 3.8	1.8 - 2.3	+	ND
		Merluccius paradoxus	Deep-water Cape hake	MSDS	69.6 - 110.4	6.96 - 11.04	1.3 - 1.6	+	0.96
				PC	93.6 - 127.2	9.36 - 12.72	1.8 - 2.1	+	0.11
				SALT	43.2 - 134.4	4.32 - 13.44	1.8 - 1.9	+	0.10
				SF	820.8 - 1243.2	82.1 - 124.3	2.3 - 2.6	+	0.01
				WIZ	26.4 - 38.4	2.64 - 3.84	1.6 - 2.0	+	0.29
				PC_{500}	2373.0 - 3120.0	237.3 - 312.0	1.8 - 2.6	+	ND
				WIZ_{50}	24.0 - 43.2	2.4 - 4.3	1.6 - 2.0	+	ND
Clupeiformes	Clupeidae	*Sardinops sagax*	Pilchard (South American pilchard)	MSDS	182.4 - 216.0	18.2 - 21.6	1.5 - 1.7	+	0.21
				PC	196.8 - 237.6	19.7 - 23.8	1.6 - 2.0	+	0.22
				SALT	26.4 - 38.4	2.6 – 3.8	1.5 - 2.8	+	0.26
				SF	1291.2 - 1756.8	129.1 - 175.7	2.3 - 2.4	+	0.02
				WIZ	21.6 - 38.4	2.2 - 3.8	1.8 - 2.2	+	0.31
				PC_{500}	2520.0 - 3120.0	252.0 - 312.0	1.6 - 2.1	+	ND
				WIZ_{50}	21.6 - 45.6	2.2 - 4.6	1.8 - 2.2	+	ND
	Engraulidae	*Engraulis japonicus*	Cape anchovy (Japanse anchovy)	MSDS	103.2 - 163.2	10.3 - 16.3	1.9 - 2.1	+	0.13
				PC	405.6 - 487.2	40.6 - 48.7	1.9 - 3.9	+	0.45
				SALT	86.4 - 165.6	8.6 - 16.5	1.9 - 3.6	+	0.13
				SF	1921.2 - 1536.0	192.1 - 153.6	2.0 - 2.2	+	0.01
				WIZ	21.6 - 40.8	2.2 - 4.8	1.7 - 1.8	+	0.38
				PC_{500}	3450.0 - 3890.0	345.0 - 389.0	1.9 - 2.3	+	ND
				WIZ_{50}	23.0 - 37.4	2.3 - 3.7	1.7 - 2.0	+	ND
Pleuronecti-formes	Soleidae	*Austroglossus pectoralis*	East coast (mud) sole	MSDS	429.6 - 508.8	43.0 - 50.9	1.5 - 1.8	+	0.50
				PC	283.2 - 341.4	28.3 - 34.2	1.7 - 2.1	+	0.28
				SALT	213.6 - 276.0	21.4 - 27.6	3.8 - 4.8	+	0.24
				SF	513.6 - 768.0	51.4 - 76.8	2.1 - 2.7	+	0.06
				WIZ	24.0 - 43.2	2.4 - 4.3	1.8 - 2.0	+	0.36
				PC_{500}	1908.0 - 2304.4	190.8 - 230.4	1.8 - 2.4	+	ND
				WIZ_{50}	12.0 - 38.4	1.2 - 3.8	1.8 - 2.0	+	ND
Scorpaen-iformes	Triglidae	*Chelidonichthys capensis*	Cape gurnard	MSDS	261.6 - 326.4	26.2 - 32.6	1.4 - 1.7	+	0.30
				PC	348.0 - 480.0	34.8 - 48.0	2.0 - 3.2	+	0.43
				SALT	453.6 - 518.4	45.4 - 51.8	3.1 - 4.1	+	0.48
				SF	1200.0 - 1291.2	120.0 - 129.1	2.1 - 2.2	+	0.01
				WIZ	28.8 - 55.2	2.9 - 5.5	1.5 - 2.0	+	0.48
				PC_{500}	3408.0 - 4080.0	340.8 - 408.0	2.0 - 2.6	+	ND
				WIZ_{50}	24.0 - 55.2	2.4 - 5.5	1.5 - 2.0	+	ND
Siluriformes	Clariidae	*Clarias gariepinus*	North African catfish	MSDS	69.6 - 96.0	7.0 - 9.6	1.2 - 1.4	+	0.09
				PC	211.2 - 240.0	21.1 - 24.0	1.7 - 2.3	+	0.22
				SALT	110.4 - 151.2	11.0 - 15.1	1.7 - 2.0	+	0.13
				SF	1435.2 - 1872.0	143.5 - 187.2	2.0 - 2.3	+	0.02
				WIZ	38.4 - 96.0	3.8 - 9.6	1.9 - 2.3	+	0.77
				PC_{500}	1728.0 - 1992.0	172.8 - 199.2	1.8 - 2.0	+	ND
				WIZ_{50}	50.4 - 96.0	5.0 - 9.6	1.9 - 2.3	+	ND

Abbreviations: MSDS = modified urea-SDS-proteinase K method; PC = phenol-chloroform method; SALT = salt extraction method; SF = SureFood PREP Allergen kit; WIZ = Wizard Genomic DNA Purification kit; LOD = lowest limit of detection; ND = not determined.

Non-shaded areas represent values obtained with methods performed according to protocols. Shaded areas represent values obtained with methods adapted for comparison. PC_{500} = PC method using 500 mg starting material. WIZ_{50} = WIZ method using 50 mg starting material.

Table 2 Separation principles employed by each DNA extraction method evaluated in this study

DNA extraction method	Principles of DNA separation
1. Modified urea-SDS-proteinase K (MSDS) method	Cell lysis at elevated temperatures using anionic detergent (SDS), chaotropic agent (urea) and protein degrading enzyme (proteinase K). Precipitation of protein with salt (NaCl) and organic solvents (phenol/chloroform). DNA precipitation with isopropanol.
2. Phenol-chloroform (PC) method	Cell lysis at elevated temperatures using anionic detergent (SDS), and protein degrading enzyme (proteinase K). DNA purification with phenol, chloroform and isopropanol. DNA precipitation with ethanol.
3. Salt extraction (SALT) method	Cell lysis at elevated temperatures using anionic detergent (SDS) and protein degrading enzyme (proteinase K). Precipitation of proteins with salt (NaCl) followed by precipitation of DNA with isopropanol.
4. SureFood® PREP Allergen kit (SF)	Cell lysis at elevated temperatures using protein degrading enzyme (proteinase K). Adjustment of optimal binding conditions, binding of DNA in a resin-containing spin column, followed by elution of DNA from the spin column.
5. Wizard® Genomic DNA Extraction kit (WIZ)	Cell lysis with detergent, RNase treatment, precipitation of proteins with salt (NaCl)-containing buffer; concentration of DNA and desalting using isopropanol.

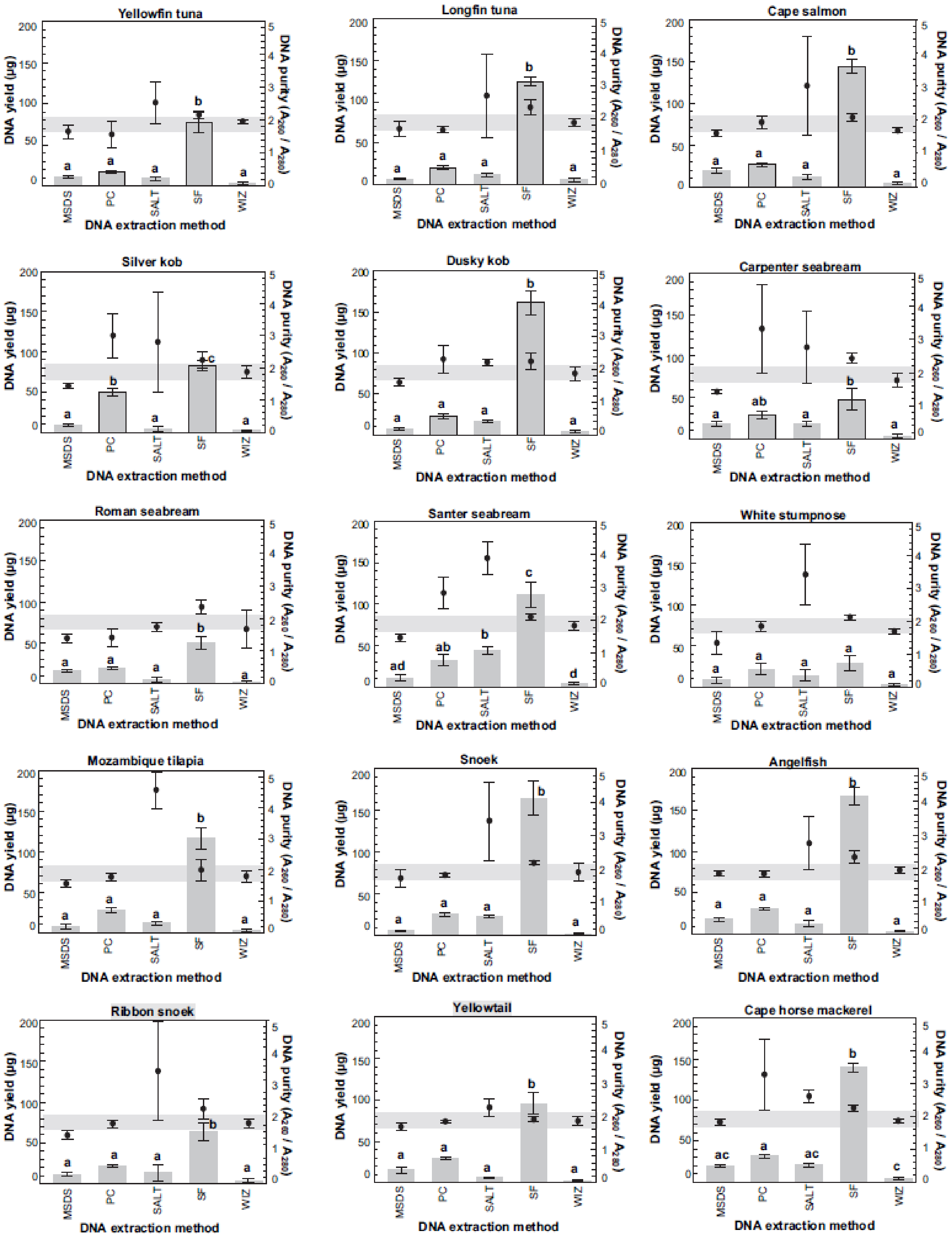


Figure 1 Comparison of DNA yields and purities obtained with five different methods, considered separately for 29 fish species. Statistically significant (P < 0.05) differences between DNA yields are indicated by non-identical subscript letters. Grey shaded areas indicate the range of purity values considered satisfactory for pure DNA (A_{260} / A_{280} of 1.7 - 2.1). Error bars indicate standard deviations from three independent replicates.

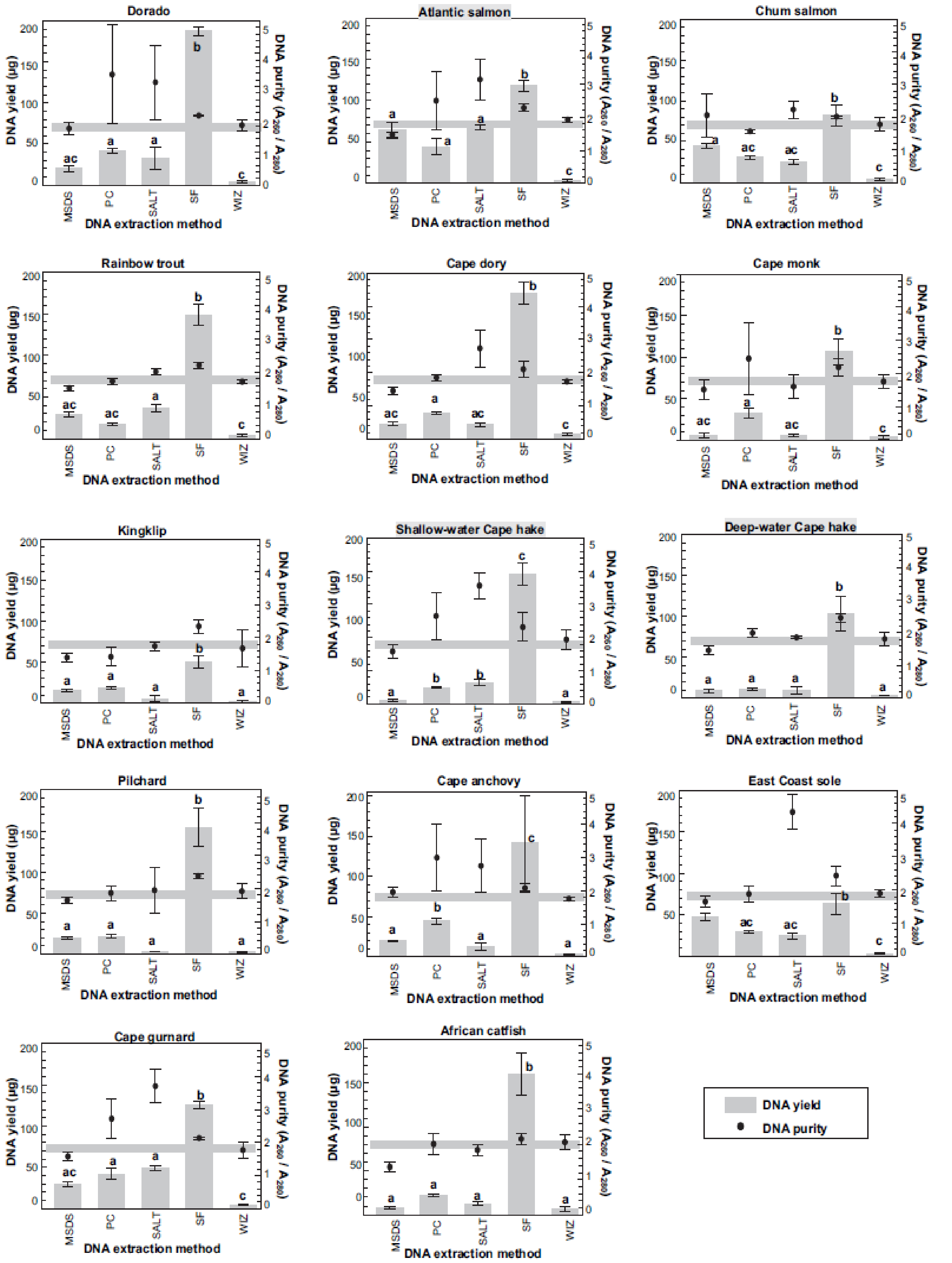
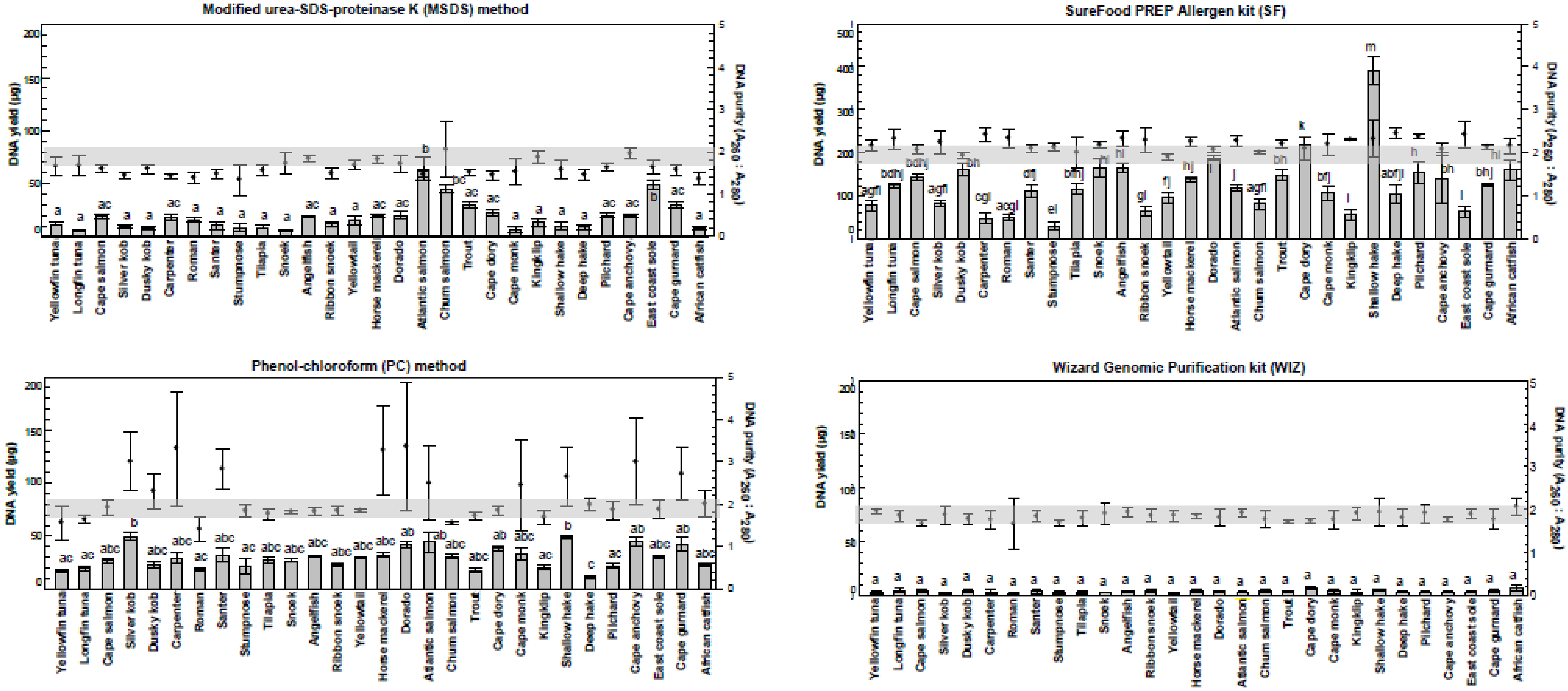


Figure 1 (Continued)



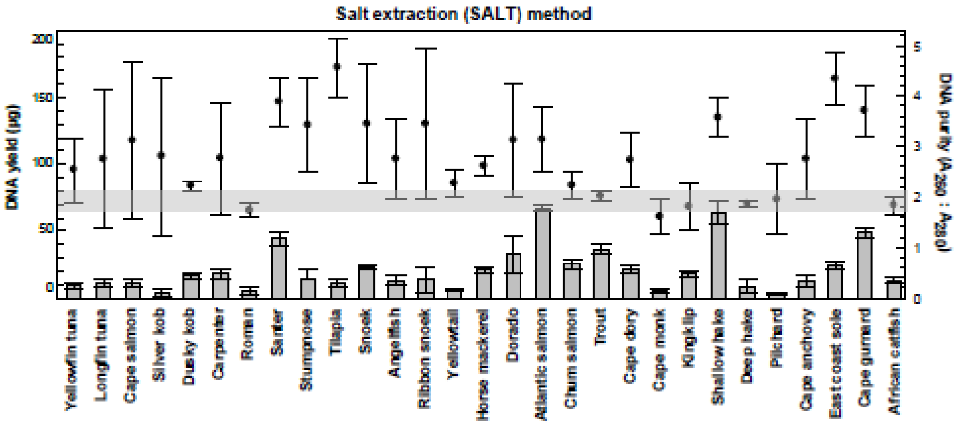
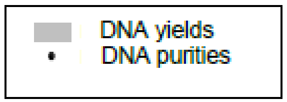


Figure 2 Comparison of the yields and purities of DNA extracted from 29 fish species, considered separately for the five different DNA extraction methods utilised. Statistically significant (P < 0.05) differences between DNA yields are indicated by non-identical subscript letters. Grey shaded areas indicate the range of purity vales considered satisfactory for pure DNA. Error bars indicate standard deviations from three independent replicates.



For the carpenter seabream, the DNA yields obtained with the SF method were significantly ($P < 0.05$) higher than those obtained with the MSDS, SALT and WIZ methods, but were not significantly ($P > 0.05$) different to the yields obtained with the PC method. The DNA yields obtained from the white stumpnose samples were relatively low using all of the extractions methods (< 40 µg), and there was no significant ($P > 0.05$) difference in the yields obtained with all five methods for this species. For the east coast sole, the DNA yields obtained with the SF method were significantly higher than those obtained with the PC, SALT and WIZ methods (Fig. 1), but were not significantly ($P < 0.05$) higher than those obtained with the MSDS method.

Table 1 shows that the WIZ method consistently extracted the lowest yields of DNA from all fish species when compared to the other DNA extraction methods. The WIZ method utilised a lower amount of starting material (20 mg fish muscle) for DNA extraction than that required by the MSDS, PC and SALT methods (50 mg) and the SF method (500 mg fish muscle). Statistically, the yields of DNA extracted with the WIZ method were significantly ($P < 0.05$) lower than the yields extracted with the SF method in 28 of the 29 (97%) fish examined, the exception being the white stumpnose.

With 14 of the 29 fish species (48%) (yellowfin tuna, longfin tuna, Cape salmon, dusky kob, roman seabream, tilapia, snoek, angelfish, ribbon snoek, yellowtail, kingklip, deep-water Cape hake, pilchard and catfish), there was no significant difference ($P > 0.05$) between the DNA yields extracted with the MSDS, PC, SALT and WIZ methods, although the DNA yields extracted with the SF method were significantly ($P < 0.05$) higher. For the remainder of the fish, some differences in the DNA extraction efficiency of the MSDS, PC, SALT and WIZ methods are observable in Fig. 1. The yields of DNA extracted from the silver kob and anchovy, for instance, were significantly ($P < 0.05$) higher with the PC method than with the MSDS, SALT and WIZ methods. For the Cape horse mackerel, Cape dory and Cape monk, the DNA yields obtained with the PC method were significantly ($P < 0.05$) higher than those obtained with the WIZ method, although these did not differ significantly ($P > 0.05$) from those yields extracted with the MSDS and SALT methods. The SALT method allowed the extraction of significantly ($P > 0.05$) higher DNA yields from the santer seabream and rainbow trout than did the WIZ method, but these did not differ significantly ($P > 0.05$) from the DNA yields extracted with the PC method. Significantly ($P < 0.05$) higher DNA yields were extracted with the PC and SALT methods from the dorado, shallow-water Cape hake and Cape gurnard than with the WIZ method. For the shallow water-Cape hake, however, the DNA yields extracted with the PC and SALT methods were also significantly ($P < 0.05$) higher than those obtained with the MSDS method. The MSDS method delivered significantly ($P < 0.05$) higher DNA yields from the chum salmon and east coast sole than did the WIZ method, but these did not differ significantly ($P < 0.05$) from the yields extracted with the PC and SALT methods (Fig. 1).

It is clear that, even when the same DNA extraction method was utilised, considerable differences were found in the DNA yields from the individual fish species (Fig. 2). Thus, it appears likely that the nature or composition of the muscle tissue of the different species may have had an effect on the ability to extract DNA from fish tissue, with certain methods being more suitable for certain species than others. It has been previously reported that the extraction of lower DNA yields can be associated with samples with higher fat content (Saunders & Rossi, 2008), although from a review of the scientific literature relating to the fat content of fish species, this did not appear to be the case in this study.

Due to the considerable variability seen in the DNA yields obtained from individual fish species with the same method, a better idea of the overall efficiency of each method may be obtained by studying the Bootstrap means plot for the DNA yields (Fig. 3). This plot summarises the DNA yield data from each method by ignoring the interactions between individual fish species and by taking the DNA yields obtained from individual fish species merely as independent replicates of a specific method. The Bootstrap means plot (Fig. 3) confirms that, overall, the SF method produced significantly ($P < 0.05$) the highest DNA yields of the five extraction methods when these were carried out according to the protocols. However, if the standard deviation between the three independent replicates is considered, the SF method appears to be the least reproducible of the five methods in terms of the yields. Nonetheless, the DNA concentrations and yields obtained with the SF method, on average, were consistently higher than 1000 $ng.ml^{-1}$ and 100 μg, respectively. Therefore, reproducibility is perhaps secondary to the ability of a method to extract high yields, as the DNA extracted with the SF method could be easily diluted to workable concentrations, and would be suitable for a wide range of molecular applications. For example, both conventional and real-time PCR protocols require template DNA concentrations of between 1 - 5 $ng.\mu l^{-1}$ (Saunders & Rossi, 2008). If the average DNA concentrations obtained with each method are considered, this would imply that the DNA extracted with the SF method would be sufficient for up to 1250 PCR reactions, while that DNA extracted with the WIZ method would be sufficient, at the best, for 30 PCR reactions.

Of the three published DNA extraction methods (MSDS, PC and SALT) using 50 mg starting material, the PC method delivered significantly ($P < 0.05$) higher DNA yields than the MSDS and SALT methods. When ignoring the interactions between individual fish species, the Bootstrap means confirm that significantly ($P < 0.05$) the lowest yields of DNA were extracted from fish muscle tissue with the WIZ method (Fig. 3). The low standard deviation seen between the three replicates with the WIZ method indicates that this method was the most reproducible in terms of the DNA yields it delivered. The suitability of this method for the extraction of DNA from fish muscle tissue would consequently depend on whether the user requires a method that can consistently deliver similar DNA yields (high reproducibility) or whether a method that delivers high DNA yields is required.

The average DNA yields obtained from fish muscle tissue of 182 µg, 288 µg, 210 µg, 1246 µg and 37 µg with the MSDS, PC, SALT, SF and WIZ methods, respectively, corresponded well with reports in the literature on DNA yields extracted from other animal tissues. Biase *et al*. (2002) reported DNA yields of 72 µg extracted from swine muscle tissue and Thumber (2002) reported DNA yields of 100 - 900 µg extracted from the muscle tissue of cattle, buffalo, sheep, goat, pig and chicken using solvent DNA extraction protocols.

DNA purities with methods carried out according to protocols

The purities of the DNA extracted from the fish species with the different extraction methods, which were estimated by calculation of the A_{260} to A_{280} ratios, are summarised in Table 1 and Fig. 2. In the case of the DNA purities, statistically significant differences between the fish species with the different extraction methods were not determined. Instead, the DNA was considered to be satisfactorily pure when the ratio of the A_{260} to A_{280} readings were within the range of 1.7 – 2.1 (Aljanabi & Martinez, 1997; Rapley, 2000; Wasko *et al*., 2003; Ferrara *et al*., 2006; Lopera-Barrero *et al*., 2008). This range is indicated by the grey shaded region in Fig. 2.

The DNA extraction methods evaluated varied in their ability to extract DNA of a suitable purity (Fig. 2). The proportion of the fish DNA extracts obtained with the five different extraction methods that fell within, below and above the range considered satisfactory for pure DNA are indicated in Table 3. The majority (72 %) of the DNA purity values obtained with the MSDS method were found to be below 1.7, which could indicate that the DNA extracted with this method was contaminated to some degree. While DNA absorbs ultraviolet light maximally at a wavelength of 260 nm, proteins, on the other hand, absorb light maximally at a wavelength of 280 nm. Thus, contamination of DNA with proteins usually reduces the A_{260} to A_{280} ratio to values lower than 1.7.

Residual impurities carried over from the DNA extraction procedure, such as phenol and ethanol, are also reported to reduce the A_{260} to A_{280} ratio (Sambrook & Russell, 2001; Wilfinger *et al*., 2006). Thus, the low DNA purity values obtained with the MSDS method were most likely due to contaminating proteins or residual reagents being co-extracted with the DNA.

With the PC method, approximately half of the DNA extracts had purities falling within the satisfactory range of 1.7 – 2.1 for pure DNA (Table 3), while the remainder of the extracts mostly had purity values exceeding 2.1. The majority (76 %) of the purity values obtained for the DNA extracted with the SALT method were considerably higher than those values considered satisfactory for pure DNA. It has been reported that both the A_{260} values and the A_{260} to A_{280} ratios may be inflated as a result of the presence of contaminating RNA in a sample. This is due to the fact that both DNA and RNA absorb ultraviolet light maximally at a wavelength of 260 nm. Absorbance spectrophotometry cannot distinguish between DNA and RNA and accurate DNA quantification is, therefore, dependent on the separation of the two nucleic acids (Wilfinger *et al*., 2006). A number of DNA extraction protocols routinely employ RNase enzymes to degrade RNA. Thus, it appears that the highly elevated A_{260} to A_{280} ratios obtained with the SALT method indicated considerable contamination of the extracted DNA with RNA. These findings were in agreement with those reported by Lopera-Barrero *et al.* (2008), who found that the DNA extracted from fish fins and larvae using the SALT method without the employment of RNase showed a high presence of contaminating RNA when separated on agarose gels. The results of this study confirm the recommendations made by these authors that the use of RNase would be a necessary requirement when using the SALT method to ensure that the extracted DNA is free from RNA.

While the SF method produced the highest concentrations of DNA of the five methods, the purities of this extracted DNA were not satisfactory for all the fish DNA extracts. Approximately half of the DNA extracts obtained with the SF method were in the satisfactory range for pure DNA (Fig. 2, Table 3). The purity values for the remainder of the DNA extracts obtained with the SF method were slightly higher than those values expected for pure DNA, possibly indicating that this method did not completely eliminate contaminating RNA from some of the DNA samples. On the other hand, while the WIZ method produced the lowest DNA yields of the five methods, all of the DNA extracts obtained with this method (100 %) were in the satisfactory range for pure DNA.

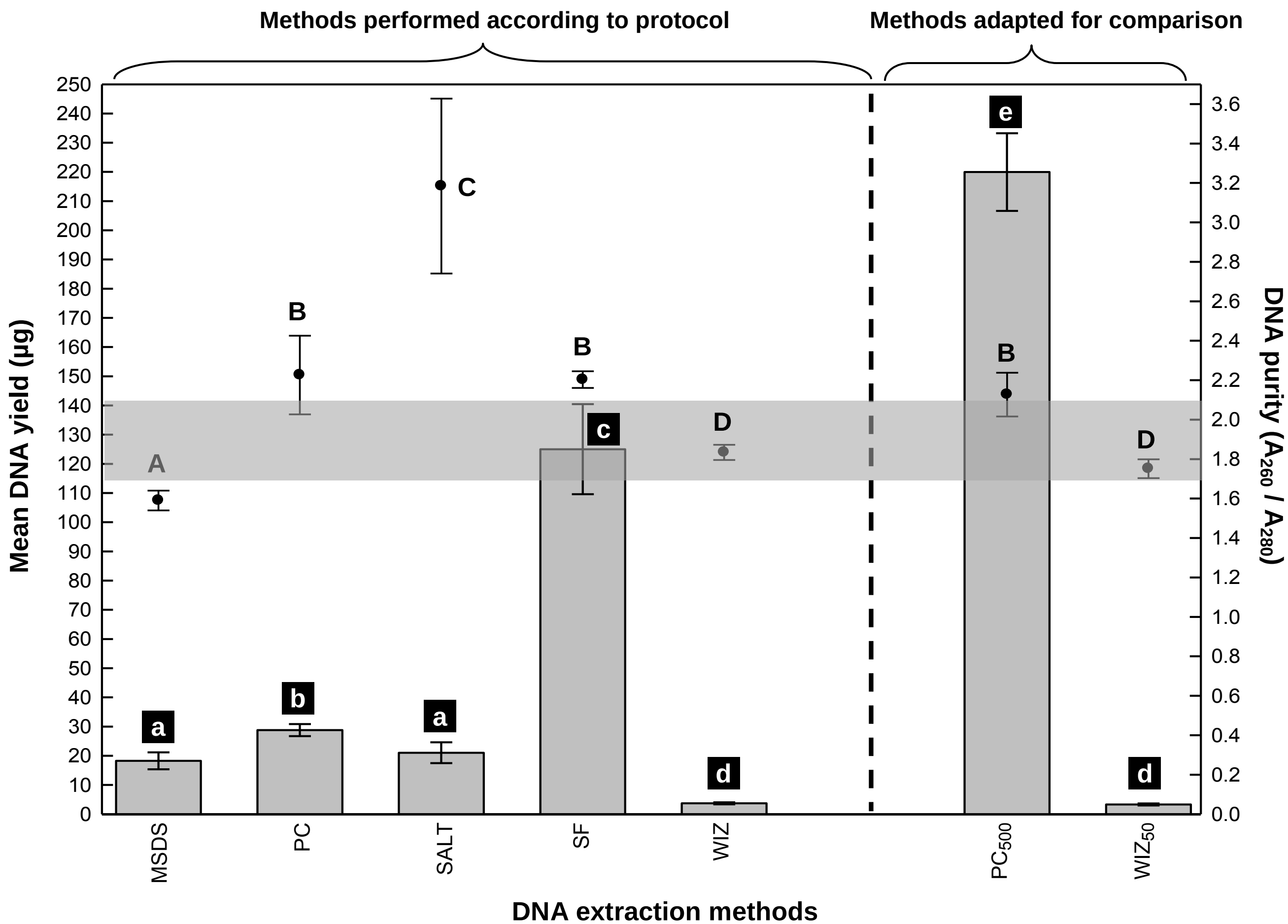


Figure 3 Bootstrap means plot showing the mean yields (vertical bars) and mean purities (scatter plots) of DNA extracted with each method when taking fish species as independent replicates (3 x 29 replicates). Statistically significant (P < 0.05) differences between mean DNA yields are indicated by non-identical lower-case subscript letters, whereas statistically significant (P < 0.05) differences between mean DNA purities are indicated by non-identical upper-case subscript letters. Grey shaded areas indicate the range of purity values considered satisfactory for pure DNA (A_{260} / A_{280} of 1.7 – 2.1). Error bars indicate standard deviations between replicates. The dashed line separates the values obtained with methods when performed according to the protocols and those values obtained with methods adapted for comparison. PC_{500} = PC method using 500 mg starting material. WIZ_{50} = WIZ method using 50 mg starting material.

Table 3 The proportion of DNA extracts from 29 fish species obtained with five different methods falling within, below and above the satisfactory range for pure DNA

	DNA extraction methods				
Purity range	**MSDS**	**PC**	**SALT**	**SF**	**WIZ**
Samples within purity range of 1.7 – 2.0	8 (28%)	14 (48%)	6 (21%)	15 (52%)	29 (100%)
Samples below purity of 1.7	21 (72%)	4 (14%)	1 (3%)	0 (0%)	0 (0%)
Samples above purity of 2.0	0 (0%)	11 (38%)	22 (76%)	14 (48%)	0 (0%)
	29 (100%)	29 (100%)	29 (100%)	29 (100%)	29 (100%)

Ignoring the interactions between individual fish species, calculation of the Bootstrap means of the DNA purities (Fig. 3) shows that, overall, the purities of the DNA extracted with the MSDS were lower, and those obtained with the PC, SALT and SF methods were higher, than those values expected for pure DNA. Overall, when ignoring the interactions between individual fish species, the data in Fig. 3 confirms that the WIZ method was the only method able to extract DNA of suitable purity (purity values of 1.7 – 2.1). This was also the only method that employed the RNase enzyme as part of the DNA extraction protocol. These results illustrate the value of utilising this enzyme to reduce RNA contamination of DNA extracted from fish muscle tissue.

Suitability of DNA for PCR amplification

PCR was employed to evaluate the quality and suitability of the extracted DNA for molecular applications. In spite of the range of yields and purities observed for the DNA extracted with the different extraction methods, all five methods delivered DNA that could be successfully amplified with the cyt *b* gene targeting PCR method (Table 1). However, when using the DNA extracted with the MSDS, PC, SALT and WIZ methods, the PCR was optimised using a volume of 3 µl of DNA template in the reaction mixture, while these volumes of DNA extracted with the SF method were found to inhibit the PCR. It is known that the PCR may be inhibited by both too low and too high concentrations of DNA in the reaction (Saunders & Rossi, 2008). Given that the concentrations of DNA extracted with the SF method were considerably higher than those concentrations derived with the other methods, it was assumed that the reason for this PCR inhibition was due to the DNA concentrations being too high, and that smaller quantities of the SF-extracted DNA would be required for successful PCR amplification. Consequently, PCR amplification of the DNA extracted with the SF method was successfully optimised using a reduced volume of 2 µl of DNA template in the reaction mixture. This observation once again illustrates that while methods that extract high concentrations and yields of DNA are advantageous from the viewpoint of providing template for a great number of applications, it is imperative that the concentration of such DNA be tailored to the specific application for which it is destined.

Limit of detection of PCR

The cyt *b* gene PCR method was shown to be highly sensitive, being capable of detecting less than 1 $pg.ul^{-1}$ of DNA regardless of the method used for DNA extraction (Table 1). The PCR, however, did show the greatest sensitivity when DNA extracted with the SF method was used for amplification, with the limit of detection (LOD) of the PCR being less than 0.1 $pg.ul^{-1}$ for all fish species (Table 1).

Standardisation of DNA extraction methods

Recognition of the fact that different amounts of starting material could have led to different final DNA yields, attempts were made to evaluate the DNA extraction methods using equivalent amounts of fish muscle tissue for DNA extraction. The PC method, which was the published method delivering the greatest yields when carried out according to the protocol (Fig. 3), was consequently repeated using 500 mg of fish muscle tissue as starting material. This was in order to allow direct comparison with the SF method and to evaluate whether the higher DNA yields obtained with the SF method were as a result of the superior extraction efficiency of this method or merely due to a higher amount of starting material being used. The concentrations, yields and purities of the DNA extracted from each fish species with the standardised PC method (PC_{500}) are presented in Table 1. The DNA yields extracted with the PC_{500} method are graphically illustrated in Fig. 3, taking all fish as independent replicates with the extraction method. This plot shows that, overall, the DNA yields extracted from fish muscle with the PC_{500} method were significantly ($P < 0.05$) higher than those DNA yields extracted with both the PC and SF methods. These results suggest that the PC method is capable of delivering higher DNA yields than those obtained with the SF method if the amount of starting materials is increased. These findings also suggest that the reason for the SF method originally extracting the highest yields of DNA from the fish samples (Table 1, Fig. 3) was more likely associated with the fact that this method employed the greatest amount of starting material than it was associated with a superior extraction efficiency of the method.

In order too evaluate whether the low DNA yields obtained with the WIZ method were attributed to this method employing the smallest amount of starting material, the WIZ method was repeated using 50 mg of fish muscle tissue as starting material. This was the equivalent amount of starting material to that used for the MSDS, PC and SALT methods when carried out according to the protocol. The concentrations, yields and purities of the DNA extracted from each fish species with the standardised WIZ method (WIZ_{50}) are presented in Table 1. The DNA yields extracted with the WIZ_{50} method are graphically illustrated in Fig. 3, taking all fish as independent replicates with the extraction method. It is clear from this plot that increasing the amount of starting material used for the WIZ method did not lead to higher mean DNA yields being extracted from fish muscle tissue. On the contrary, the mean DNA yields extracted from 50 mg of starting material with the WIZ method (WIZ_{50}) tended to be lower than those obtained from 20 mg of fish muscle, although these differences were not statistically significant ($P > 0.05$) (Fig. 3). Thus, the significantly ($P < 0.05$) lower DNA yields observed for the WIZ method in Fig. 3 would still apply even in the case when the amount of starting material used for this method was increased to be in line with that used for the MSDS, PC and SALT methods (50 mg).

The mean DNA purities obtained with the WIZ_{50} method tended to be lower than those purities obtained with WIZ method performed according to the protocol (Fig. 3). Nonetheless, the mean purities from both the WIZ_{50} and WIZ methods were in the range considered satisfactory for pure DNA.

Comparison of the feasibility of different extraction methods

Time and labour requirements

Overall, there were not noteworthy discrepancies in the labour and time requirements for the execution of the five DNA extraction methods, with all methods involving multiple reagent additions, incubation steps and tube transfers. The simplicity of utilising the SF and WIZ methods was, however, promoted by the fact that the majority of the reagents required for DNA extraction were included in the kits in a ready-to-use format. Thus, the commercial kits were found to be highly suitable for high throughput applications involving the extraction of DNA from a great number of samples. With the published DNA extraction protocols (MSDS, PC and SALT), the procurement and preparation of the individual reagents required by the protocols was necessary.

Economic feasibility

The advantages gained in labour and time savings with the SF and WIZ methods were offset by the greater costs of these commercial kits compared to the published protocols. A comparison of the five DNA extraction methods in terms of their relative costs is shown in Table 4. At a first glance, the SF method appears to be the most costly method when considering the cost for the extraction of DNA from individual samples. However, the high yields extracted with this method imply that the DNA extracted from a single sample could be used for a greater number of applications than the DNA extracted with any other method. Thus, the yields of DNA should be taken into account when comparing the costs of the methods.

Table 4 Comparison of the five DNA extraction methods in terms of the costs per sample and per microgram of DNA extracted

	MSDS	**PC**	**SALT**	**SF**	**WIZ**
Cost per DNA extraction	X*	1.5X	X	7.0X	3.0X
Mean DNA yield (µg) extracted	18.27	28.79	21.25	125.03	3.72
Cost per µg of extracted DNA	0.05X	0.06X	0.05X	0.05X	0.8X

* X = *ca*. 6.5 ZAR, 0.54 EUR, 0.72 USD, 0.49 GBP, 1.0 AUD, based on prevailing exchange rates

The calculation of the costs per microgram of extracted DNA indicates that the cost of the SF method was comparable with, or cheaper than, the other methods evaluated. The WIZ method was estimated to be a costly method, both for the extraction of DNA per individual sample and per microgram of DNA that this method delivered.

Safety considerations

The safety of all reagents used in the five DNA extraction methods was evaluated and compared using the material safety datasheets obtained on procurement, as well as information available in the United States Department of Labor Occupational Safety and Health Association Chemical Database (OSHA, 2009).

Although many of the reagents used for the extraction of DNA are generally classified as irritants to the eyes and skin, the use of these products is not likely to have harmful effects when they are used and handled according to the specifications. Of the five DNA extraction methods evaluated, perhaps the greatest health and safety concerns lay with the use of phenol and chloroform in the MSDS and PC methods. Both phenol and chloroform are classified as serious hazards (OSHA, 2009). Phenol is highly corrosive, causing burns to the skin after accidental exposure, and is reported to cause acute poisoning, damage to the eyes or blindness, and may indeed lead to death. Similarly, the inhalation or ingestion of chloroform is harmful to health and may be fatal. Chloroform is reasonably anticipated to be a human carcinogen and cause of reproductive damage. The aforementioned safety risks are probably one of the major reasons that many laboratories worldwide no longer use phenol-chloroform methods for the extraction of DNA. On the other hand, the use of the reagents supplied with the SF and WIZ commercial kits did not appear to pose any serious health and safety risks according to the information provided by the manufacturers.

Conclusions

To our knowledge, this is the first study that has compared the efficiency of different methods for the extraction of DNA from the muscle tissue of different fish species, in particular those species commercially available in South Africa. The results of this study indicated variability in the efficiencies of different DNA extraction methods for the extraction of high quantities of pure DNA from the muscle tissue of different fish species. The extraction of DNA that is of a high quantity and quality is generally recognised as the most important factor for the success of molecular techniques, such as PCR, PCR-RFLP and DNA sequencing. Overall, this study identified the SF method as the most suitable method for the extraction of DNA from fish muscle tissue, due to the high DNA yields delivered, the relative safety of the method, the ease of use and the applicability for high throughput extractions from multiple specimens. Although DNA yields comparable to those extracted with the SF method could be obtained by up-scaling the amount of starting material used with the PC method, the additional labour requirements and safety concerns of this method made this the secondary choice for the extraction of high DNA yields from fish muscle. While the purity of the DNA extracted with the PC and SF method was not considered to be optimal, both methods produced DNA that could be readily amplified by the PCR. Therefore, although the incorporation of a RNase enzyme or additional clean-up step could be used to improve the purity of the extracted DNA, this did not appear necessary in this study.

References

Aranishi, F. (2005). PCR-RFLP analysis of nuclear nontranscribed spacer for mackerel species identification. *Journal of Agricultural and Food Chemistry*, **53**, 508-511.

Aranishi, F. (2006). Single fish egg DNA extraction for PCR amplification. *Conservation Genetics*, **7**, 153-156.

Aljanabi, S.M. & Martinez, I. (1997). Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Research,* **25***,* 4692-4693.

Bardakci, F. & Skibinski, D.O. (1994). Application of the RAPD technique in tilapia fish: species and subspecies identification. *Heredity*, **73**, 117-123.

Bartlett, S.E. & Davidson, W.S. (1992). FINS (Forensically informative nucleotide sequencing): a procedure for identifying the animal origin of biological specimens. *BioTechniques*, **12**, 408-411.

Biase, F.H., Franco, M.M., Goulart, L.R. & Antunes, R.C. (2002). Protocol for extraction of genomic DNA from swine solid tissues. *Genetics and Molecular Biology*, **25**, 313-315.

Bickley, J., Short, J.K., McDowell, D.G. & Parkes, H.C. (1996). Polymerase chain reaction (PCR) detection of *Listeria monocytogenes* in diluted milk and reversal of PCR inhibition caused by calcium ions. *Letters in Applied Microbiology*, **22,** 153-158.

Cėspedes, A., García, T., Carrera, E., González, I., Sanz, B., Hernández, P.E. & Martin, R. (1998). Identification of flatfish species using polymerase chain reaction (PCR) amplification and restriction analysis of the cytochrome *b* gene. *Journal of Food Science*, **63**, 206-209.

Cėspedes, A., García, T., Carrera, E., González, I., Fernández, A., Hernández, P.E. & Martin, R. (1999). Identification of Sole *(Solea solea)* and Greenland Halibut *(Reinhardtius hippoglossoides)* by PCR Amplification of the 5S rDNA Gene. *Journal of Agricultural and Food Chemistry*, **47**, 1046-1050.

Chakraborty, A., Sakai, M. & Iwatsuki, Y. (2006). Museum fish specimens and molecular taxonomy: a comparative study on DNA extraction protocols and preservation techniques. *Journal of Applied Ichthyology*, **22**, 160-166.

Chapela, M.J., Sotelo, C.G., Pérez-Martín, R.I., Pardo, M.Á., Pérez-Villareal, B., Gilardi, P. & Riese, J. (2007). Comparison of DNA extraction methods from muscle of canned tuna for species identification. *Food Control*, **18**, 1211-1215.

Comi, G., Lacumin, L., Rantsiou, K., Cantoni, C. & Cocolin, L. (2005). Molecular methods for the differentiation of species used in production of cod-fish can detect commercial frauds. *Food Control*, **16**, 37-42.

Di Pinto, A., Forte, V., Guastadisegni, M.C., Martino, C., Schena, F.P. & Tantillo, G. (2007). A comparison of DNA extraction methods for food analysis. *Food Control*, **18**, 76-80.

FAO (Food and Agriculture Organization of the United Nations) (2009). *The state of world fisheries and aquaculture*. Pp. 1-153. Rome, Italy: FAO.

Ferrara, G.B., Murgia, B., Parodi, A.M., Valisano, L., Cerrano, C., Palmisano, G., Bavestrello, G. & Sara, M. (2006). The assessment of DNA from marine organisms via a modified salting-out protocol. *Cellular & Molecular Biology Letters*, **11**, 155-160.

Kakihara, Y., Matsufuji, H., Chino, M. & Takeda, M. (2006). Extraction and detection of endogenous soybean DNA from fermented foods. *Food Control*, **17**, 808-813.

Lockley, A.K. & Bardsley, R.G. (2000). DNA-based methods for food authentication. *Trends in Food Science and Technology,* **11**, 67-77.

Lopera-Barrero, N.M., Povh, J.A., Ribeiro, R.P., Gomes, P.C., Jacometo, C.B. & Da Silva Lopes, T. (2008). Comparison of DNA extraction protocols of fish fin and larvae samples: modified salt (NaCl) extraction. *Ciencia e Investigación Agraria*, **35**, 65-74.

Lucentini, L., Caporali, S., Palomba, A., Lancioni, H. & Panara, F. (2006). A comparison of conservative DNA extraction methods from fins and scales of freshwater fish: a useful tool for conservation genetics. *Conservation Genetics*, **7**, 1009-1012.

Mackie, I.M. (1996). Authenticity of fish. In: *Food Authentication* (edited by P.R. Ashurt & M.J. Dennis). Pp. 140-170. London, UK: Blackie Academic and Professional.

Mafra, I., Silva, S.A., Moreira, E.J.M.O., Ferreira da Silva, C.S., Beatriz, M. & Oliveira, P.P. (2008). Comparative study of DNA extraction methods for soybean derived food products. *Food Control*, **19**, 1183-1190.

Marmiroli, N., Peano, C. & Maestri, E. (2003). Advanced PCR techniques in identifying food components. In: *Food Authenticity and Traceability* (edited by M. Lees). Pp. 3-33. Cambridge, UK: Woodhead Publishing Ltd.

Meyer, R., Hofelein, C., Luthy, J. & Candrian, U. (1995). Polymerase chain reaction-restriction fragment polymorphism analysis: a simple method for species identification in food. *Journal of AOAC International*, **78**, 1542-1551.

Pardo, M.A. & Pérez-Villarreal, B. (2004). Identification of commercial canned tuna species by restriction site analysis of mitochondrial DNA products obtained by nested primer PCR. *Food Chemistry,* **86**, 143-150.

OSHA (Occupational Safety and Health Association) (2009). United States Department of Labor OSHA/EPA Occupational Chemical Database. [Internet document]. URL www.osha.gov/web/dep/chemicaldata/#target. 10 July 2009.

Peano, C., Samson, M.C., Palmieri, L., Gulli, M. & Marmiroli, N. (2004). Qualitative and quantitative evaluation of the genomic DNA extracted from GMO and non-GMO foodstuffs with four different extraction methods. *Journal of Agricultural and Food Chemistry*, **52**, 6962-6968.

Rapley, R. (2000). Molecular analysis and amplification techniques. In: *Molecular Biology and Biotechnology* (edited by J.M. Walker & R. Rapley). Pp. 27-29. London, UK: Royal Society of Chemistry.

Ren, X., Zhu, X., Warndorf, M., Bucheli, P. & Shu, Q. (2006). DNA extraction and fingerprinting of commercial rice cereal products. *Food Research International*, **39**, 433-439.

Rijpens, N.P., Jannes, G., Van Asbroeck, M., Rossau, R. & Herman, L.M.F. (1996). Direct detection of *Brucella* spp. in raw milk by PCR and reverse hybridization with 16S-23S rRNA spacer probes. *Applied and Environmental Microbiology*, **62**, 1683-1688.

Sackesen, C. & Adalioglu, G. (2003). Hidden fish substance triggers allergy. *Journal of Investigational Allergology and Clinical Immunology*, **13**, 216-217.

Sambrook, J. & Russel, D. W. (2001). *Molecular Cloning: A Laboratory Manual*, 3rd ed. Pp. 6-15. New York, US: Cold Spring Harbor Laboratory Press.

Saunders, G.C. & Rossi, J.M. (2008). DNA extraction. In: *Essentials of Nucleic Acid Analysis: A Robust Approach* (edited by J.T. Keer & L. Birch). Pp. 59-64. Great Britain, UK: Royal Society of Chemistry.

StatSoft, Inc. (2006). STATISTICA (data analysis software system), version 7.1. [Internet document]. URL http// www.statsoft.com. 10 March 2009.

Thumber (2002). Meat speciation by molecular and serological techniques. MSc Thesis. Anand Agricultural University, Western India.

Wasko, A.P., Martins, C., Oliveira, C. & Foresti, F. (2003). Non-destructive genetic sampling in fish. An improved method for DNA extraction from fish fins and scales. *Hereditas*, **138**, 161-165.

Wilfinger, W. Mackey, K. & Chomczynski, P. (2006). Assessing RNA and DNA following purification. In: *DNA Sequencing II: Optimizing the Preparation and Clean-up* (edited by J. Kieleczawa). Pp. 295-308. Boston, US: Jones & Bartlett Publishers.

Wilson, I.G. (1997). Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology*, **63**, 3741-3751.

Yue, G.H. & Orban, L. (2001). Rapid isolation of DNA from fresh and preserved fish scales for polymerase chain reaction. *Marine Biotechnology*, **3**, 199-204.

CHAPTER 5

EVALUATION OF THE 16S AND 12S rRNA GENES AS UNIVERSAL MARKERS FOR THE IDENTIFICATION OF COMMERCIAL FISH SPECIES IN SOUTH AFRICA

Abstract

The development of DNA-based methods for the identification of fish species is important for fisheries research and control, as well as for the detection of unintentional or fraudulent species substitutions in the marketplace. The aim of this study was to generate a comprehensive reference database of DNA sequences from the mitochondrial 16S and 12S ribosomal RNA (rRNA) genes for 53 commercial fish species in South Africa and to evaluate the applicability of these genetic markers for the identification of fish at the species level. The DNA extracted from all target species was readily amplified by the polymerase chain reaction (PCR) using universal primers targeting both rRNA gene regions. Sequences from the 16S rRNA and 12S rRNA genes were submitted to GenBank for the first time for 34% and 53% of the fish species, respectively. Cumulative analysis of the 16S rRNA gene sequences revealed mean conspecific, congeneric and confamilial Kimura two parameter (K2P) distances of 0.03%, 0.70% and 5.10% and the corresponding values at the 12S rRNA gene level were 0.03%, 1.00% and 5.57%. K2P neighbour-joining trees based on both sequence datasets generally clustered species in accordance with their current taxonomic classifications. The nucleotide variation existing in both the 16S rRNA and 12S rRNA gene sequences was suitable for identifying the large majority of the examined fish specimens to at least the level of genus, but was found to be less useful for the explicit differentiation of certain congeneric fish species. It is therefore recommended that one or more faster-evolving DNA regions be analysed to confirm the identities of closely-related fish species in South Africa.

Cawthorn, D.M., Steinman, H.A. & Witthuhn, R.C. (2012). Evaluation of the 16S and 12S rRNA genes as universal markers for the identification of commercial fish species in South Africa. *Gene*, **491**(1), 40-48.

Introduction

Accurate species identification is central to fisheries research and management, but is a challenge throughout the life cycle of fishes, from eggs and larvae to fingerling and adult stages (Kochzius *et al*., 2010). The United Nations Food and Agriculture Organization (FAO) has recognised species identification as a significant fisheries management issue since the 1960s. Aggregate data from 1950 to 2002 indicate that around 21% of global fish catches failed to be identified to the species level and species resolution in capture fisheries is reportedly decreasing (Lleonart *et al*., 2006). Concurrent with these trends is the increasing concern relating to overfishing and the consequent decline of marine biodiversity around the world, the erroneous identification of overexploited species and the threats of illegal, unreported and unregulated (IUU) fishing on conservation strategies (Pauly *et al*., 2005; Ogden, 2008; FAO, 2009). Additionally, marine resource scarcity has led to the recent widespread proliferation of fraudulent market substitutions of high-valued fish with those of lower value, which has been facilitated by the globalisation of seafood trade and the fact that many fish products are traded in the processed form (Jacquet & Pauly, 2008). All these factors, combined with a dwindling pool of taxonomists focused on morphological species identifications, signal the need for the development of accurate analytical methods for the authentication of a wide variety of fish species (Buyck, 1999; Schander & Willassen, 2005).

There is now growing consensus within the scientific community that genetic methods, particularly those based on direct DNA sequence analysis, can compliment traditional morphological examinations, resolving questions relating to the taxonomic identity of specimens at different life-history stages and providing a vital tool for exposing fraud and illegal species trading (Tautz *et al*., 2003, Comi *et al*., 2005; Hajibabaei *et al*., 2007). Although regions of both the nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) can be analysed for species identification purposes, the mtDNA has preferably been used for such applications in both forensic and non-forensic fields (Bartlett & Davidson, 1992; Comi *et al*., 2005; Chauhan & Rajiv, 2010; Linacre & Tobe, 2011). Vertebrate mtDNA is maternally inherited, not subject to the diversity-generating sexual reassortment occurring in nDNA, and its rapid mutation rate leads to the accumulation of sufficient point mutations to allow the differentiation of even closely-related species (Vawter & Brown, 1986; Kocher *et al*., 1989). In addition, the high copy number of mtDNA in each cell, which exceeds that of nDNA several fold, minimises the possibility of amplification failure due to low DNA concentrations with degraded templates (Robin & Wong, 1989; Tobe & Linacre, 2008). Among the most frequently used mtDNA markers for the identification of vertebrates are the cytochrome *b* (cyt *b*) gene (Kocher *et al*., 1989; Céspedes *et al*., 1998; Parson *et al*., 2000), cytochrome *c* oxidase I (COI) gene (Hebert *et al*., 2003; Ward *et al*., 2005; Vences *et al*., 2005) and the 16S and 12S rRNA genes (Prakash *et al*., 2000; Girish *et al*., 2004; Rastogi *et al*., 2004; 2007; Melton & Holland, 2007; Mitani *et al*., 2009).

The success of a universal DNA marker for species identification relies on portions of the chosen gene being sufficiently conserved for PCR primer annealing, but also on the sequence amplified between the primers to be adequately species-specific to allow for differentiation (Vences *et al*., 2005; Karlsson & Holmlund, 2007). Since it is known that mtDNA presents a degree of intra-species variability, it is also vital to select a DNA marker that exhibits considerably less intra- than inter-species variation if unambiguous identifications are to be made (Lockley & Bardsley, 2000; Gharrett *et al*., 2001). The 16S and 12S rRNA genes are relatively conserved genes, evolving more slowly than the mitochondrial genome as a whole (Palumbi, 1996; Di Finizio *et al*., 2007). The highly conserved regions of these loci can be used as primer-binding sites, while the mutations existing in the variable regions reportedly make both genes suitable for species discrimination (Balitzki-Korte *et al*., 2005; Vences *et al*., 2005). Partial regions of the mitochondrial rRNA genes have been selected as standard markers for phylogeny reconstruction in amphibians and the 16S rRNA gene has, in fact, been shown to be superior to the COI gene for amphibian species identifications (Vences *et al*., 2005). While numerous studies have employed partial sequences of the 16S and 12S rRNA genes to discriminate between groups of fishes (Akimoto *et al*., 2002; Comesaña *et al*., 2003; Ishizaki *et al*., 2006; Di Finizio *et al*., 2007; Zhang *et al*., 2007; Ardura *et al*., 2010), relatively few have assessed the intra- and inter-species variation shown in these regions. Information on the intra- and inter-species variation in the COI and cyt *b* genes of vertebrates have been published (Su *et al*., 1999; Ward *et al*., 2005; Rock *et al*., 2008; Lakra *et al*., 2011) and the availability of such data for the 16S and 12S rRNA genes would permit greater confidence to be associated with 100% species matches, while aiding in interpreting lower percentage matches and determining if sequence divergences may be due to intra-species variation (Linacre & Tobe, 2011).

The coastal waters surrounding South Africa are inhabited by over 2 200 different fish species (13% of which are endemic) and the country represents the most important fisheries role player on the African continent (Van der Elst, 1997; INFOSA, 2007). A variety of domestically-caught species are sold on the South African market (Cawthorn *et al*., 2011), however, as many of these have increasingly become the targets of overfishing, more foreign fish species have become available to compensate the local demand for fishery products. The development of reliable methods for fish species authentication has consequently become a research priority in South Africa for both fisheries management and for the control of commercial practices. Partial 16S rRNA sequencing was recently employed by Von der Heyden *et al*. (2010) for the identification of certain fish species in South Africa. Nonetheless, the results emerging from this work were confounded in some cases by a lack of reference 16S rRNA gene sequences in GenBank for a number of fish species encountered in this country. In fact, a search of GenBank reveals that reference DNA sequence data is limited or absent for numerous commercially significant fish species in South Africa (e.g. kingklip, kabeljou species and west coast sole), which complicates identification regardless of the molecular marker chosen for analysis. The aim of this study was to generate a comprehensive database of reference 16S and 12S rRNA sequences for 53 fish species commercially available in South Africa, to assess the intra- and inter-species variation in these rRNA gene loci and to evaluate the overall applicability of these regions in permitting the unambiguous identification of fish species.

Materials and Methods

Sampling

Fifty three commercially important fish species from 42 genera in 23 families of ray-finned fishes (*Actinopterygii*) were obtained from prominent fishing companies, seafood processors and the Department of Agriculture, Forestry and Fisheries (DAFF) in South Africa (Table 1). Most of the collected fish were locally-caught marine species, while seven species were imported, but were included in the study as these have been shown to be commonly marketed in South Africa (Cawthorn *et al*., 2011). At least 3 specimens of each species were analysed. The numbers (N) of specimens per species ranged from 3 to 12, with a mean of 3.66 specimens per species. All fish specimens were morphologically identified to the species level by fish taxonomists. Specimens were stored at -20 °C prior to analysis.

DNA extraction and amplification

DNA was extracted from the muscle tissue excised from each fish specimen using the SureFood® PREP allergen kit (r-Biopharm, supplied by AEC-Amersham, Cape Town, South Africa) following the instructions of the manufacturer. A *ca*. 560 base pair (bp) fragment of the 16S rRNA gene was PCR amplified from all DNA extracts using the universal primers 16SarL (5'-CGC CTG TTT ATC AAA AAC AT-3') and 16SbrH (5'-CCG GTC TGA ACT CAG ATC ACG T-3') (Palumbi, 1996). This primer set has been applied in the past for the identification of amphibians and fish species (Akimoto *et al*., 2002; Vences *et al*., 2005; Ishizaki *et al*., 2006; Von der Heyden *et al*., 2010). The 25 µl PCR reaction mixtures contained 2.5 µl (1 X) reaction buffer ($MgCl_2$ free) (Super-Therm, supplied by Southern Cross Biotechnologies, Cape Town, South Africa), 2.5 µl (2.5 mM) $MgCl_2$ (25 mM, Super-Therm), 0.3 µl (0.12 µM) of each primer (10 µM stocks), 0.20 µl (1.0 U) *Taq* DNA polymerase (5U/µl, Super-Therm), 0.25 µl (0.1 mM) of mixed dNTPs (10 mM, AB gene, supplied by Southern Cross Biotechnologies) and 1 µl (*ca*. 1 µg) of DNA template. The PCR cycling conditions used were as follows: initial denaturation at 94 °C for 2 min, 30 cycles of denaturation at 93 °C for 30 s, primer annealing at 55 °C for 40 s and chain elongation at 72 °C for 60 s, followed by final extension at 72 °C for 5 min.

The universal primers 12S1 (5'-GAC AGC TAC GAC ACA AAC TGC GAT TAG ATA CC-3') and 12S2 (5'-TGC ACC TTC CAG TAC ACT TAC CAT GTT ACG AC-3') (Infante *et al*., 2006) were utilised to amplify a *ca*. 543 bp fragment of the 12S rRNA gene from all fish specimens. The reaction mixtures (25 µl final volume) comprised 2.5 µl (1 X) reaction buffer ($MgCl_2$ free) (Super-Therm), 2.0 µl (2.0 mM) $MgCl_2$ (25 mM, Super-Therm), 0.5 µl (0.20 µM) of each primer (10 µM stock solutions), 0.25 µl (1.25 U) *Taq* DNA polymerase (5U/µl, Super-Therm), 0.25 µl (0.1 mM) of dNTPs (10 mM, AB gene) and 1 µl (*ca*. 1 µg) of DNA template. The thermal cycling regime included an initial denaturation step at 96 °C for 2 min, 35 cycles of 96 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s, followed by a final extension step of 72 °C for 10 min. All PCR amplifications were performed in a Mastercycler Personal (Eppendorf, Germany). PCR products were separated by electrophoresis (90 volts for 45 min) in 1.5% (m/v) agarose (Sigma-Aldrich, Gauteng, South

Africa) gels and were visualised under an ultraviolet light (Vilber Lourmat, Marne La Vallee, France).

DNA sequencing and sequence analysis

PCR products were purified with the NucleoFast 96 PCR Clean-up Kit (Macherey-Nagel, supplied by Separations, Gauteng, South Africa) according to the manufacturer's instructions. The purified PCR products were sequenced on an ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, USA) using the PCR amplification primers as sequencing primers. The generated 16S and 12S rRNA gene sequences were aligned using the complete alignment application in Clustal X version 2.0 (Larkin *et al*., 2007). Intra-species and inter-species sequence divergence values, hereafter referred to as distance or D, were calculated with the Kimura two parameter (K2P) distance model (Kimura, 1980) using MEGA version 4.0 (Tamura *et al*., 2007). Neighbor-joining (NJ) trees were constructed in MEGA 4 with the pairwise deletion of missing nucleotide data option. The robustness of topology nodes were evaluated using the Bootstrap method with 1000 replications (Felsenstein, 1985). All generated DNA sequences were submitted to GenBank (accession numbers in Table 1) and were also compared to those sequences already available in GenBank using the nucleotide basic local alignment search tool (BLASTn) algorithm.

Results and discussion

Common names, taxonomic designations, as well as GenBank accession numbers for all fish specimens sequenced for the 16S and 12S rRNA gene regions are shown in Table 1. The DNA extracted from all 53 fish species was readily amplified with the universal 16S and 12S rRNA gene targeting primers, producing a total of 380 sequences due to the analysis of multiple specimens of each species. New DNA sequence data were generated for numerous commercially important fish species in South Africa that were previously unavailable in GenBank. For 23% of the fish species under study, this was the first time that DNA sequence data of any kind were made available in GenBank. Sequences from the 16S rRNA and 12S rRNA genes were submitted to GenBank for the first time for 34% and 53% of the fish species, respectively (Table 1).

Table 1 Species, sample sizes (N), origins and GenBank accession numbers of specimens sequenced, with grey shading indicating the first entries into GenBank for each gene region

Order	Family	Species	Common name * (Local name)	N	Country of origin	16S rRNA gene GenBank accession No.	12S rRNA gene GenBank accession No.
Clupeiformes	Clupeidae	*Clupea harengus*	Atlantic herring	3	North sea	HQ592201 - HQ592203	HM003565 - HM003567
		Sardinops sagax	South American pilchard (pilchard/sardine)	3	South Africa	HQ592233 - HQ592235	HQ592301 - HQ592303
	Engraulidae	*Engraulis encrasicolus*	European anchovy (anchovy)	3	South Africa	HQ592221 - HQ592223	HM003559 - HM003561
		Engraulis japonicus	Japanese anchovy (Cape anchovy)	3	South Africa	HQ592224 - HQ592226	HM003562 - HM003564
Gadiformes	Merlucciidae	*Merluccius capensis*	Shallow-water Cape hake	4	South Africa	GU946587 - GU946589, HQ592194	GU946492 - GU946494, HQ641692
		Merluccius paradoxus	Deep-water Cape hake	7	South Africa	GU946582 - GU946586, HQ641672, HQ641673	GU946488 - GU946491, HQ641689 - HQ641691
Lophiiformes	Lophiidae	*Lophius vomerinus*	Devil anglerfish (Cape monk)	3	South Africa	GU946656 - GU946658	HM003543 - HM003545
Ophidiiformes	Ophidiidae	*Genypterus blacodes*	Pink cusk-eel (ling)	3	New Zealand / Argentina	HQ592251, HQ592252, HQ592253	HQ592313, HQ592314, HQ592315
		Genypterus capensis	Kingklip	12	South Africa	GU946612 - GU946619, HQ641676 - HQ641679	GU946514 - GU946517
Perciformes	Bramidae	*Brama brama*	Atlantic pomfret (angelfish)	4	South Africa	HQ592217 - HQ592220	HQ592272, HQ592273, HQ592276, HQ641702
	Carangidae	*Seriola lalandi*	Yellowtail amberjack	4	South Africa	GU946609 - GU946611, HQ592197	HM003546 - HM003549
		Seriola quinqueradiata	Japanese amberjack	3	China	HQ592195, HQ592196, HQ641683	HQ592270, HQ592271, HQ641706
		Trachurus capensis	Cape horse mackerel (maasbanker)	3	South Africa	GU946665 - GU946667	GU946545 - GU946547

Table 1 (continued)

Order	Family	Species	Common name * (Local name)	N	Country of origin	16S rRNA gene GenBank accession No.	12S rRNA gene GenBank accession No.
Perciformes	Coryphaenidae	*Coryphaena hippurus*	Common dolphinfish (dorado)	4	South Africa	GU946620 - GU946622, HQ641686	GU946501 - GU946503, HQ641693
	Centrolophidae	*Centrolophus niger*	Rudderfish (black ruff)	3	South Africa	GU946674 - GU946676	GU946554 - GU946556
		Schedophilus velaini	Violet warehou (black butterfish)	3	South Africa	HQ592236 - HQ592238	HQ592280 - HQ592282
		Seriolella brama	Common warehou	3	Australia / New Zealand	HQ592207 - HQ592209, HQ592210	HM003531, HM003532, HQ641697, HM003533
	Epigonidae	*Epigonus telescopus*	Black cardinal fish (cardinal)	3	South Africa	GU946597 - GU946599	GU946498 - GU946500
	Gempylidae	*Lepidocybium flavobrunneum*	Escolar (butterfish)	3	South Africa	GU946641- GU946643	GU946551 - GU946553
		Ruvettus pretiosus	Oilfish (butterfish)	3	South Africa	GU946644 - GU946646	GU946518 - GU946520
		Thyrsites atun	Snoek	6	South Africa / New zealand	GU946628, HQ592199, HQ592200, GU946626, GU946627, HQ641680	HQ592283 - HQ592285, HQ641703 - HQ641705
	Istiophoridae	*Makaira nigricans*	Blue marlin	3	South Africa	HQ592242 - HQ592244	HQ592310 - HQ592312
		Tetrapturus angustirostris	Shortbill spearfish	3	South Africa	HQ592257 - HQ592259	HQ592286 - HQ592288
	Sciaenidae	*Argyrosomus inodorus*	Mild meagre (silver kob)	7	South Africa	GU946600 - GU946605, HQ641675	GU946504 - GU946509, HQ641694
		Argyrosomus japonicus	Japanese meagre (dusky kob)	3	South Africa	GU946606 - GU946608	GU946510 - GU946512
		Atractoscion aequidens	Geelbek croaker (geelbek/Cape salmon)	5	South Africa	GU946593 - GU946596, HQ641674	HM003550 - HM003552, HQ641699, HQ641700

Table 1 (continued)

Order	Family	Species	Common name * (Local name)	N	Country of origin	16S rRNA gene GenBank accession No.	12S rRNA gene GenBank accession No.
Perciformes	Scombridae	*Gasterochisma melampus*	Butterfly kingfish (gastora)	3	South Africa	GU946623 - GU946625	GU946521 - GU946523
		Katsuwonus pelamis	Skipjack tuna	3	South Africa	HQ592230 - HQ592232	HQ592295 - HQ592297
		Scomber japonicus	Chub mackerel	3	South Africa	HQ592254 - HQ592256	HQ592277 - HQ592279
		Scomberomorus commerson	Spanish mackerel (king mackerel/couta)	3	South Africa	HQ592263 - HQ592265	HM003556 - HM003558
		Thunnus alalunga	Albacore (longfin tuna)	3	South Africa	GU946662 - GU946664	GU946542 - GU946544
		Thunnus albacares	Yellowfin tuna	4	South Africa	GU946659 - GU946661, HQ641685	HM003553 - HM003555, HQ641701
		Thunnus obesus	Bigeye tuna	3	South Africa	HQ592266 - HQ592268	HQ592316 - HQ592318
	Serranidae	*Cephalopholis sonnerati*	Tomato hind (tomato rockcod)	3	South Africa	HQ592260 - HQ592262	HQ592298 - HQ592300
		Epinephelus marginatus	Dusky gouper (yellowbelly rockcod)	3	South Africa	HQ592227 - HQ592229	HQ641707 - HQ641709
	Sparidae	*Argyrozona argyrozona*	Carpenter seabream (silverfish)	3	South Africa	GU946638 - GU946640	GU946539 - GU946541
		Cheimerius nufar	Santer seabream	4	South Africa	GU946650 - GU946652, HQ641681	GU946536 - GU946538, HQ641695
		Chrysoblephus anglicus	Englishman seabream	3	South Africa	HQ592239 - HQ592241	HQ592307 - HQ592309
		Chrysoblephus laticeps	Roman seabream	3	South Africa	GU946647- GU946649	GU946527 - GU946529
		Chrysoblephus puniceus	Slinger seabram	3	South Africa	HQ592214 - HQ592216	HQ592304 - HQ592306

Table 1 (continued)

Order	Family	Species	Common name * (Local name)	N	Country of origin	16S rRNA gene	12S rRNA gene
						GenBank accession No.	GenBank accession No.
Perciformes	Sparidae	*Pachymetopon blochii*	Hottentot seabream	3	South Africa	GU946590 - GU946592	GU946495 - GU946497
		Pterogymnus laniarius	Panga seabream	3	South Africa	HQ592211 - HQ592213	HM003537 - HM003539
		Rhabdosargus globiceps	White stumpnose	3	South Africa	GU946632 - GU946634	GU946533 - GU946535
	Trichiuridae	*Lepidopus caudatus*	Silver scabbardfish (buttersnoek/ribbon fish)	3	South Africa	GU946668 - GU946670	HM003540 - HM003542
	Xiphiidae	*Xiphias gladius*	Swordfish	3	South Africa	GU946671 - GU946673	GU946548 - GU946550
Pleuronectiformes	Soleidae	*Austroglossus microlepis*	West coast sole	6	South Africa	GU946573 - GU946577, HQ641671	GU946480 - GU946483, HQ641688, HQ641687
		Austroglossus pectoralis	Mud sole (East coast sole)	4	South Africa	GU946578 - GU946581	GU946484 - GU946487
Salmoniformes	Salmonidae	*Oncorhynchus mykiss*	Rainbow trout	3	South Africa	HQ592248 - HQ592250	HQ592289 - HQ592291
		Oncorhynchus keta	Chum salmon (Alaskan salmon)	3	USA	HQ592245 - HQ592247	HQ592292 - HQ592294
		Salmo salar	Atlantic salmon	5	Norway / Canada	GU946654, HQ641682, HQ641684 GU946653, GU946655	HM003527 - HM003529, HM003530, HQ641696
Scorpaeniformes	Sebastidae	*Helicolenus dactylopterus*	Blackbelly rosefish (jacopever)	3	South Africa	GU946635 - GU946637	GU946524 - GU946526
	Triglidae	*Chelidonichthys capensis*	Cape gurnard	3	South Africa	HQ592204 - HQ592206	GU946530 - GU946532
Zeiformes	Zeidae	*Zeus capensis*	Cape dory	3	South Africa	GU946629 - GU946631	HM003534 - HM003536

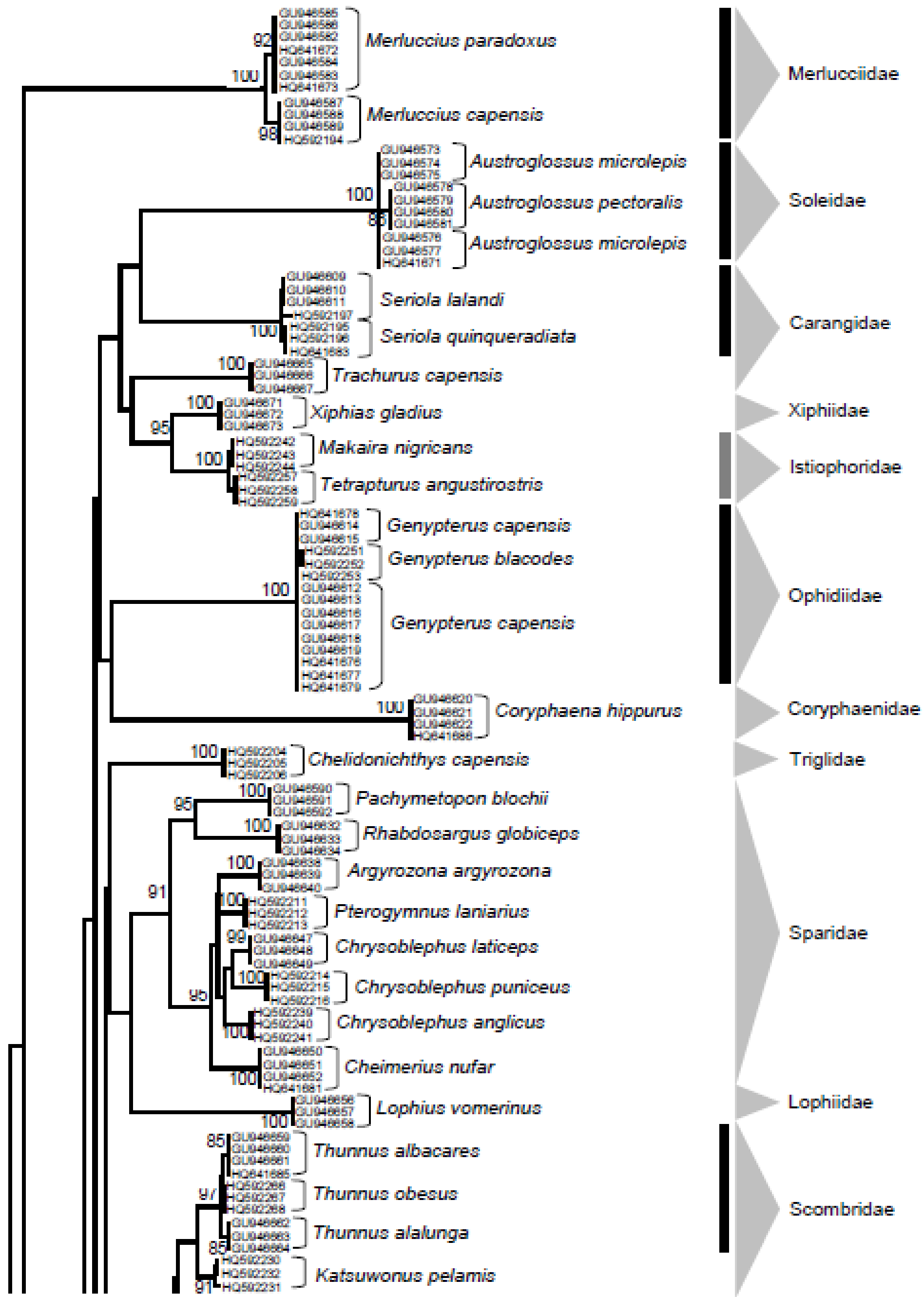
* Common names are from FishBase (www.fishbase.org)

16S rRNA sequences

The read lengths of the mitochondrial 16S rRNA gene sequences averaged 560 bp. The mean overall nucleotide base frequencies observed for these sequences were G (23.29% ± 0.06), C (24.69% ± 0.06), A (28.59% ± 0.07) and T (23.43% ± 0.07). The full K2P/NJ tree based on the 16S rRNA gene sequences is presented in Figure 1. Examination of this K2P/NJ tree indicates that members of the same species generally clustered together, as did species within genera and within families, results which are consistent with the current taxonomic classifications of the examined fish species.

The minimum, mean and maximum K2P distances (in percentages) observed in the 16S data for different taxonomic levels are provided in Table 2, the distributions of which are shown in Figure 2. Although the mean K2P distances within species, genera and families increased with taxonomic rank (Table 2, Fig. 2), the values at all taxonomic levels were relatively small, likely attributable to the highly conserved nature of the mitochondrial 16S rRNA gene in vertebrates (Di Finizio *et al*., 2007; Kitano *et al*., 2007). Studies on the faster-evolving COI gene (Ward *et al*., 2005; 2008; Steinke *et al*. 2009; Lakra *et al*. 2011) have, for instance, revealed mean intra- and inter-species K2P distances for fishes that are approximately 10-fold greater than those obtained for the 16S rRNA gene sequences. The mean intra-species variation obtained at the 16S rRNA gene level appeared particularly small (D = 0.03%) (Table 2), considerably lower than the corresponding value (D = 0.23%) reported for the partial 16S rRNA gene sequences of 50 fish species derived from eight regions of the European seas (Kochzius *et al*., 2010). Here, 48 of the 53 (*ca*. 91%) fish species showed zero intra-species variation at the 16S rRNA gene level. It should be noted, however, that sample sizes in this study were generally small and further sampling, including specimens from a wider geographic coverage, could uncover greater intra-species variation for some of the examined fish species. The maximum intra-species sequence variation (D = 0.60%) was observed between specimens of *Thyrsites atun* (snoek), which was one of few species for which both locally caught and imported specimens were analysed. Geographic differentiation is apparent for *T. atun* in Figure 1, with one clade comprising the South Africa (SA) individuals and another comprising the New Zealand (NZ) individuals, and no shared haplotypes found between the SA and NZ specimens.

It is well established that the performance of a single gene sequence in delineating and identifying species is highly dependent on the extent of separation between intra-species variation and inter-specific divergence in the selected DNA marker, often referred to as a ‘barcoding gap’ (Meyer & Paulay, 2005; Kochzius *et al*., 2010). The greater the


Merluccius paradoxus
Merluccius capensis
Austroglossus microlepis
Austroglossus pectoralis
Austroglossus microlepis
Seriola lalandi
Seriola quinqueradiata
Trachurus capensis
Xiphias gladius
Makaira nigricans
Tetrapturus angustirostris
Genypterus capensis
Genypterus blacodes
Genypterus capensis
Coryphaena hippurus
Chelidonichthys capensis
Pachymetopon blochii
Rhabdosargus globiceps
Argyrozona argyrozona
Pterogymnus laniarius
Chrysoblephus laticeps
Chrysoblephus puniceus
Chrysoblephus anglicus
Cheimerius nufar
Lophius vomerinus
Thunnus albacares
Thunnus obesus
Thunnus alalunga
Katsuwonus pelamis
Merlucciidae
Soleidae
Carangidae
Xiphiidae
Istiophoridae
Ophidiidae
Coryphaenidae
Triglidae
Sparidae
Lophiidae
Scombridae

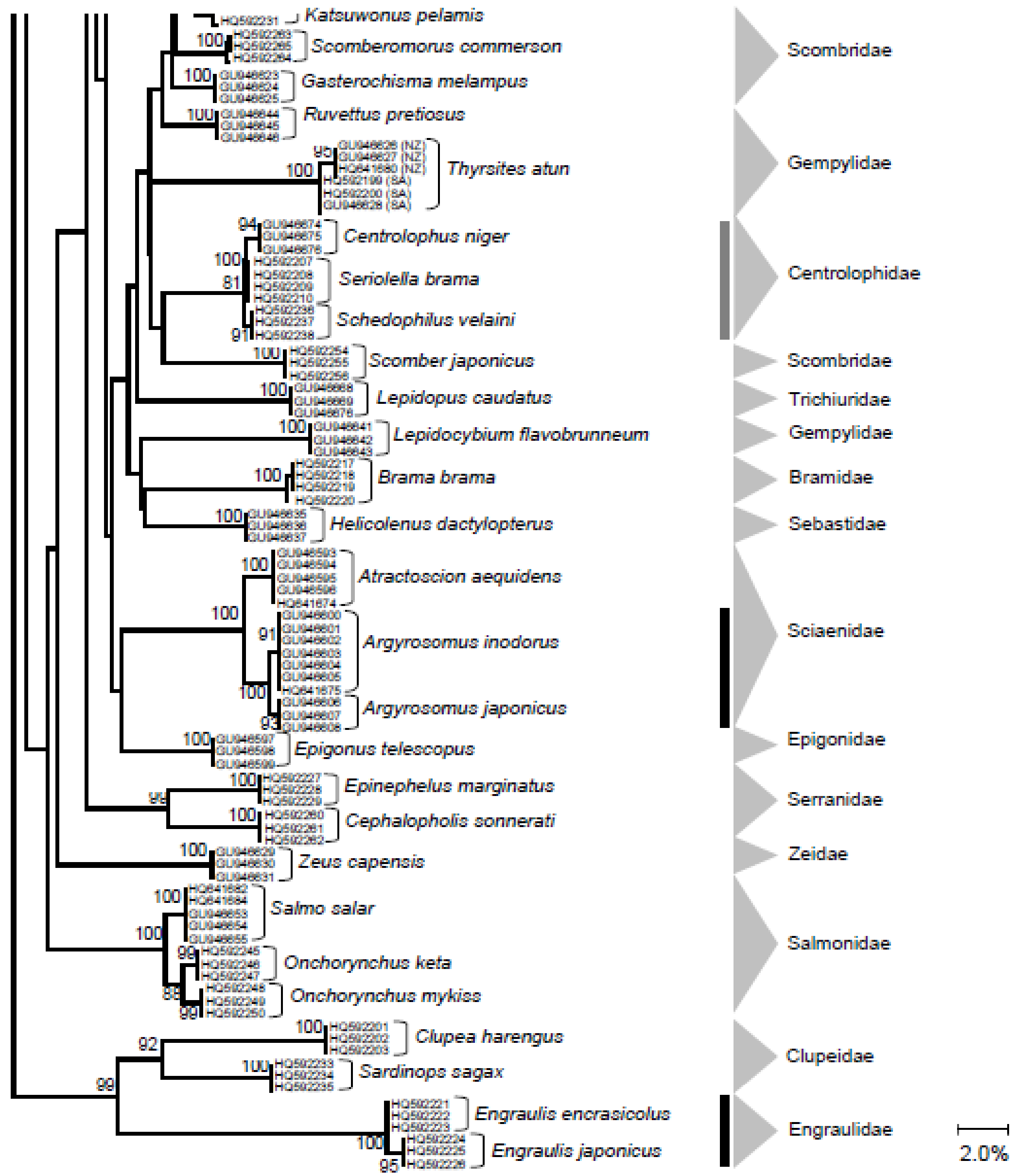


Figure 1 K2P distance neighbour-joining tree of 194 16S rRNA gene sequences from 53 fish species, with GenBank accession numbers for each specimen. Numbers at nodes indicate bootstrap values (values higher than 80 are given). Species within genera showing less than 1% sequence divergence are indicated by black bars, while species within families showing less than 1% divergence are indicated by grey bars.

Table 2 Summary of genetic divergences calculated for different taxonomic levels using K2P distances (%) with data represented for the 16S rRNA and 12S rRNA regions for 53 fish species within 43 genera and 23 families

Gene	Comparisons within	No. of sequences	Taxa	No. of comparisons	Minimum distance (%)	Mean distance (%)	Maximum distance (%)	Standard error distance (%)
16S rRNA	Species	194	53	320	0.00	0.03	0.60	0.005
	Genus	194	43	190	0.00	0.70	2.50	0.044
	Family	194	23	635	0.40	5.10	14.5	0.132
12S rRNA	Species	186	53	260	0.00	0.03	0.50	0.007
	Genus	186	53	166	0.00	1.00	2.30	0.063
	Family	186	53	635	0.00	5.57	15.20	0.133

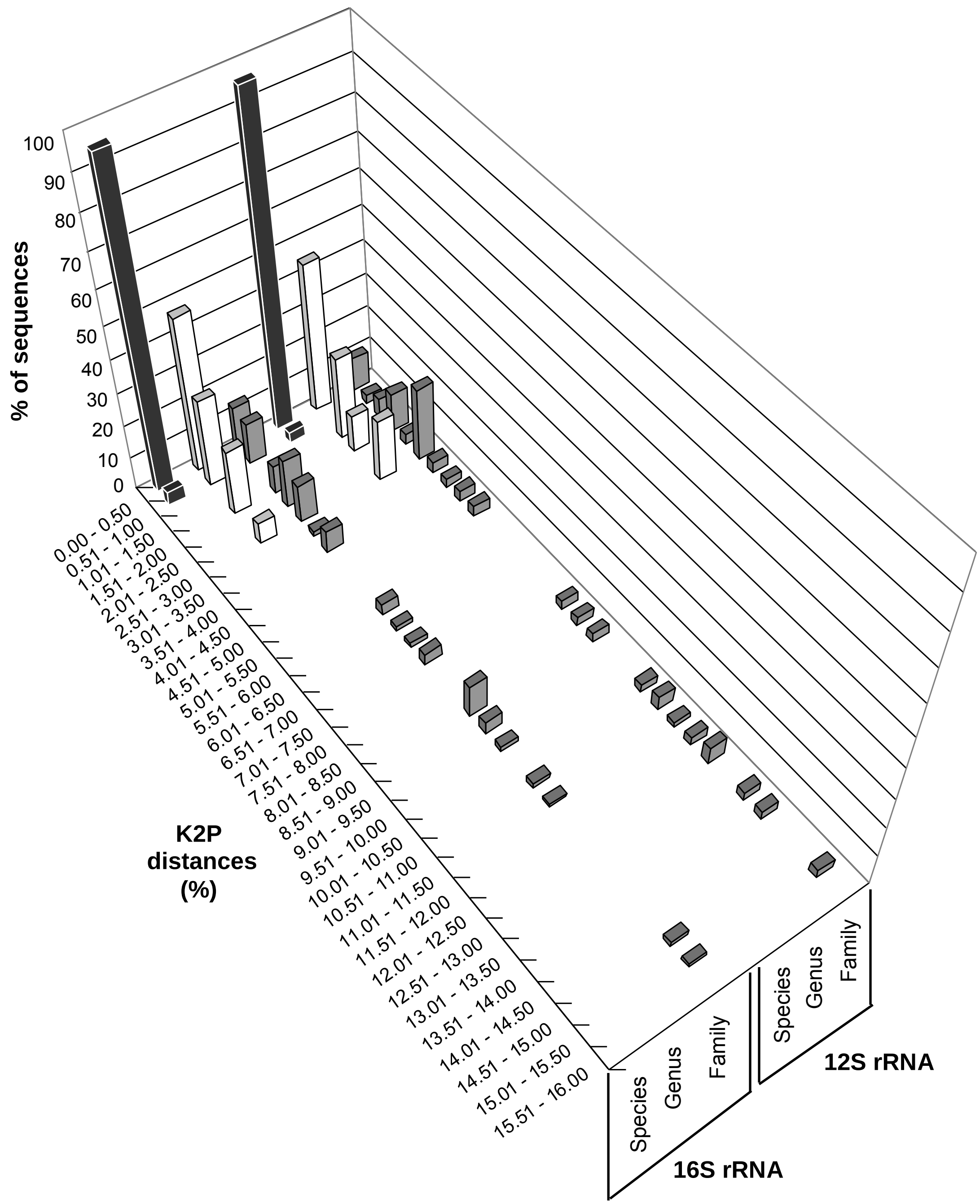


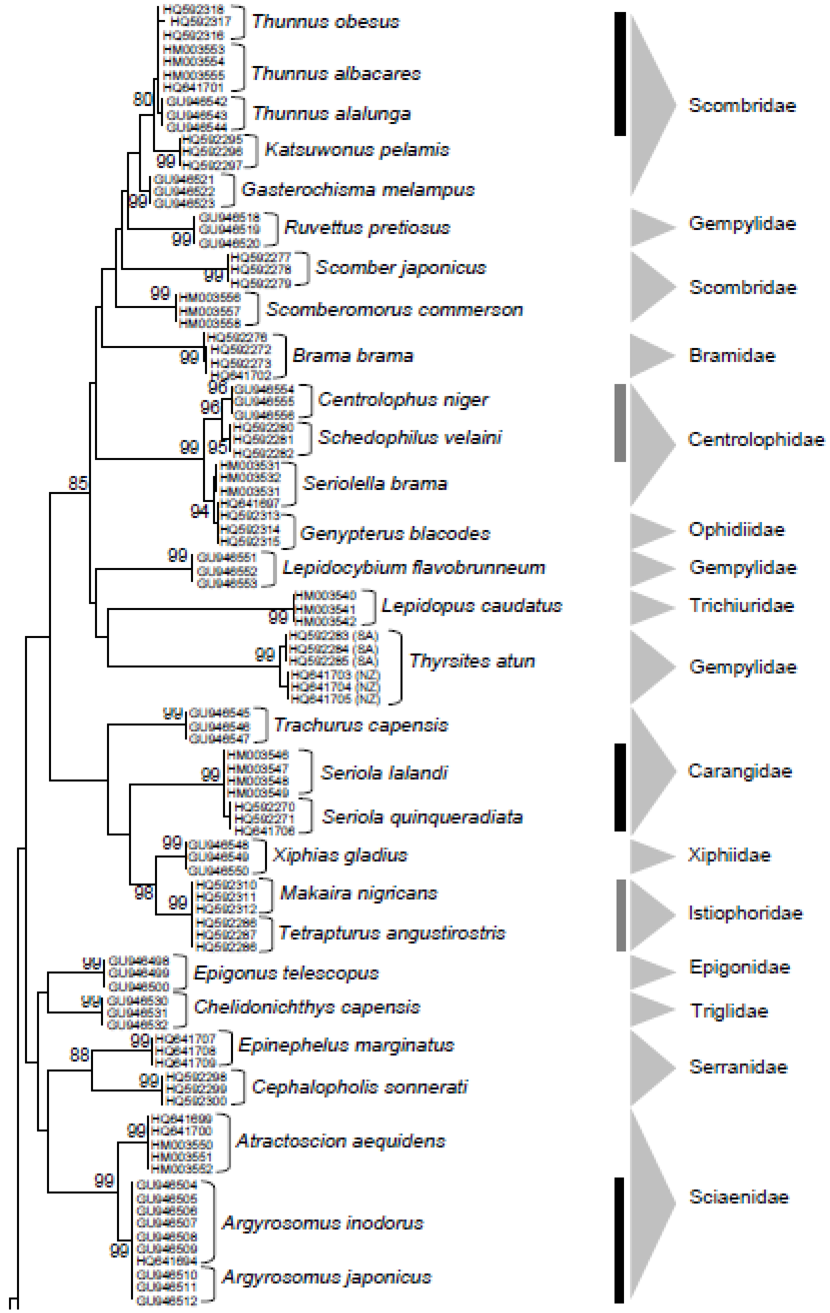
Figure 2 The distribution of K2P distances (in percentages) within various taxonomic levels for the 16S rRNA and 12S rRNA gene sequences.

overlap that exists between genetic variation within species and divergence separating species, the lower is the probability of making accurate and unambiguous species identifications. For the 16S rRNA gene sequences, the mean distance between species within genera (D = 0.70%) was *ca*. 23-fold greater than the mean distance between individuals within species (D = 0.03%) (Table 2), suggesting the existence of a 'barcoding gap'. Nonetheless, the frequency distribution of K2P distances (Fig. 2) for the 16S rRNA sequences did not support the presence of such a 'gap', since some overlap in the genetic variation at within- and between-species levels is evident from examination of this graphical representation. Such a finding indicates that the utilisation of mean genetic distance values can exaggerate the magnitude of a 'barcoding gap', as also found by Kochzius *et al*. (2010). The results obtained here show that the maximum intra-species distance (D = 0.60%) for the 16S sequences was only marginally smaller than the mean inter-species variation (D = 0.70%), and was in fact greater than the minimum congeneric distance (D = 0.0%) and minimum confamilial distance (D = 0.40%). It may be inferred from this data that intra-species variation at the 16S rRNA gene level could obscure inter-species variation in some cases and lead to confounded identifications being made based on this genetic region.

Approximately 81% of the 16S rRNA gene sequences of congeneric species showed K2P distance values lower than 1% (indicated with black bars in Figure 1) and thus the discrimination of these congeners could be problematic with this gene region. Zero divergence was seen in the sequences of *Genypterus capensis* (kingklip) and *G. blacodes* (pink cusk eel/ling), for which the lack of differentiation is also apparent from examination of the K2P/NJ tree (Fig. 1). The sequence of one specimen of *G. blacodes* was 100% identical to that of the three *G. capensis* specimens, while the sequences of the remaining two members of *G. blacodes* exhibited only single base variations with those obtained from *G. capensis*. Sequencing of the 16S rRNA gene region can thus not be expected to discriminate these two species. Other challenging cases associated with the 16S rRNA gene region involved the failure to clearly differentiate three species within the genus *Thunnus*, two *Seriola* species, two *Merluccius* species, two *Argyrosomus* species and two *Engraulis* species. Although the lack of clear resolution of closely-related fish species could be attributed to species hybridization or introgression, these are believed to be minor problems when employing mtDNA for fish species identifications (Ward *et al*., 2009), and this finding was thus more likely due to the low mutation rate in the 16S. The low sequence divergence seen among members of the genera *Thunnus* and *Seriola* correspond to those results reported by Mitani *et al*. (2009), who showed that five *Thunnus* species shared identical 16S rRNA gene haplotypes, as did two *Seriola* species, and neither of these groups could be distinguished by partial 16S rRNA gene sequencing. Comparable to the shortcomings seen in discriminating congeneric species, 7% of the 16S sequences of confamilial species exhibited K2P distances below 1% (indicated with grey bars in Figure 1). Such results suggest that 16S rRNA gene sequencing may not be suitable for discriminating the confamilial species *Makaira nigricans* (blue marlin) and *Tetrapturus angustirostris* (shortbill spearfish), neither the three species belonging to the Centrolophidae family.

12S rRNA sequences

Read lengths of the 12S rRNA gene sequences averaged 490 bp and the mean overall nucleotide base frequencies observed for these sequences were A (22.39% ± 0.06), C (25.95% ± 0.10), A (29.88% ± 0.07) and T (21.78% ± 0.07). The full K2P/NJ tree based on 12S rRNA gene sequences is presented in Figure 3, which indicates that members of the same species consistently clustered together. While species within genera and families also grouped in most cases on the basis of the 12S rRNA gene sequences, notable exceptions included the separation of members of the family Ophidiidae, as well as members of the family Gempylidae. The minimum, mean and maximum 12S K2P distances among different taxonomic levels are given in Table 2, the distributions of which are represented in Figure 2. For the 12S rRNA gene sequences, the mean inter-species distance (D = 1.00%) was found to be *ca*. 33-fold greater than the mean intra-specific distance (D = 0.03%), which was slightly higher than the corresponding ratio seen for the 16S rRNA gene sequences (Table 2). Nonetheless, the existence of a clear 'barcoding gap' was once again not supported by the frequency distribution of K2P distances (Fig. 2), which indicated an overlap in the range of intra- and inter-specific 12S rRNA gene sequence divergence, mainly attributed to the low inter-specific divergence between many congeneric species. The maximum intra-species variation (D = 0.50%) was considerably greater than the minimum congeneric (D = 0.0%) and confamilial distances (D = 0.0%) seen at the 12S rRNA gene level, indicating that ambiguous results could potentially arise for some closely-related species when using this genetic region for identification purposes. As seen for the 16S data, the maximum conspecific distance among the 12S rRNA gene



Thunnus obesus
Thunnus albacares
Thunnus alalunga
Katsuwonus pelamis
Gasterochisma melampus
Ruvettus pretiosus
Scomber japonicus
Scomberomorus commerson
Brama brama
Centrolophus niger
Schedophilus velaini
Seriolella brama
Genypterus blacodes
Lepidocybium flavobrunneum
Lepidopus caudatus
Thyrsites atun
Trachurus capensis
Seriola lalandi
Seriola quinqueradiata
Xiphias gladius
Makaira nigricans
Tetrapturus angustirostris
Epigonus telescopus
Chelidonichthys capensis
Epinephelus marginatus
Cephalopholis sonnerati
Atractoscion aequidens
Argyrosomus inodorus
Argyrosomus japonicus
Scombridae
Gempylidae
Scombridae
Bramidae
Centrolophidae
Ophidiidae
Gempylidae
Trichiuridae
Gempylidae
Carangidae
Xiphiidae
Istiophoridae
Epigonidae
Triglidae
Serranidae
Sciaenidae

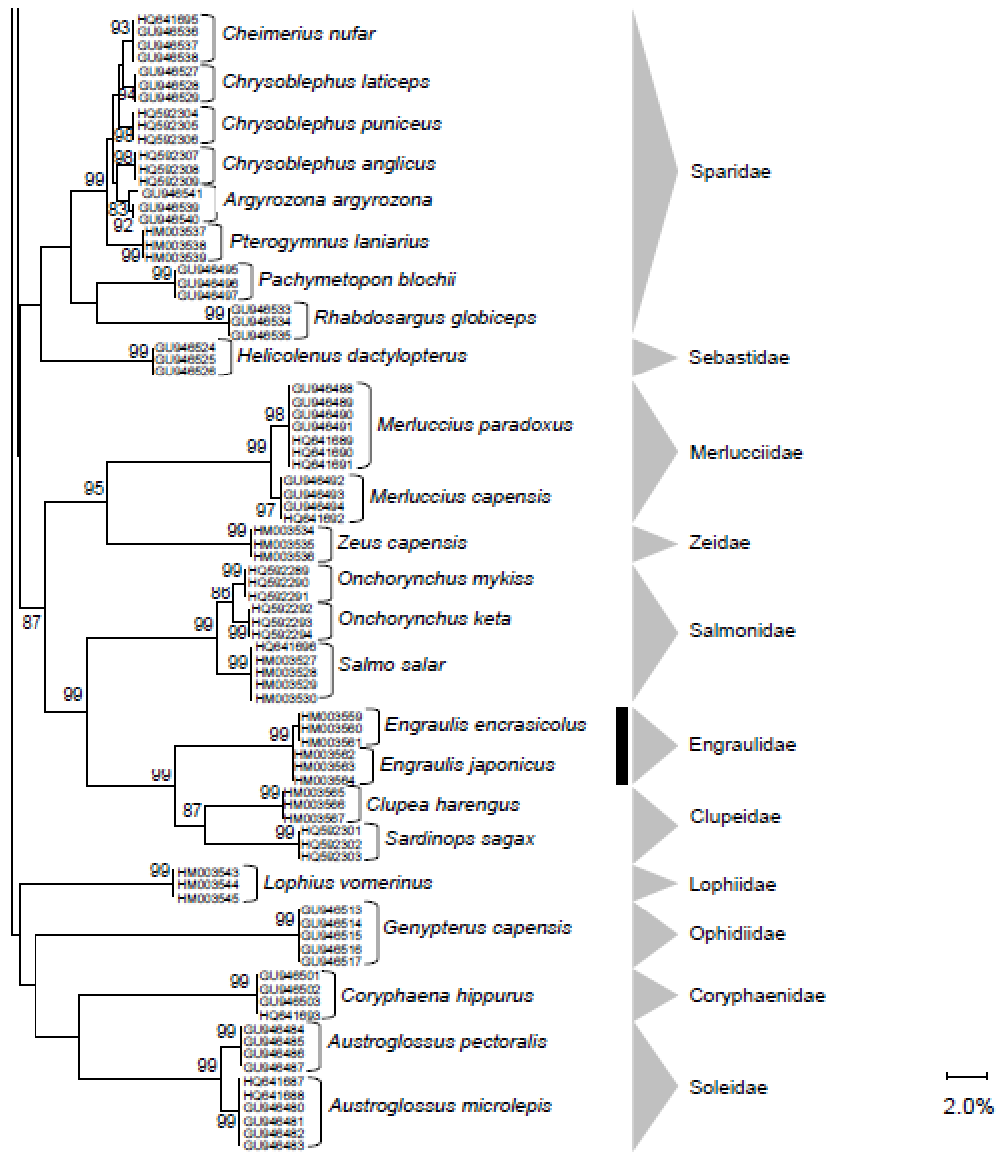


Figure 3 K2P distance neighbour-joining tree of 186 12S rRNA gene sequences from 53 fish species, with GenBank accession numbers for each specimen. Numbers at nodes indicate bootstrap values (values higher than 80 are given). Species within genera showing less than 1% sequence divergence are indicated by black bars, while species within families showing less than 1% divergence are indicated by grey bars.

sequences (D = 0.50%) was for members of *T. atun*, with the SA specimens separating at a 99% bootstrap value from the NZ specimens (Fig. 3).

Approximately 44% of the 12S rRNA gene sequences of congeneric species showed K2P distance values of less than 1%, indicated with black bars in Figure 3. Similarly, less than 1% K2P divergence was seen in 3% of the 12S rRNA gene sequences of confamilial species, indicated by grey bars in Figure 3. As was seen with the 16S rRNA gene sequences, there were cases where zero sequence divergence values were observed among the 12S rRNA gene sequences of congeneric species. Such cases occurred between *Argyrosomus inodorus* (silver kob) and *A. japonicus* (dusky kob), as well as between *T. albacares* (yellowfin tuna) and two specimens of *T. obesus* (bigeye tuna).

Also similar to the results obtained with the 16S rRNA sequences, clear differentiation did not appear feasible for a number of other congeneric species showing less than 1% divergence at the 12S rRNA level, such as *Seriola lalandi* (yellowtail amberjack) and *S. quinqueradiata* (Japanese amberjack), as well as the two *Engraulis* species. The 12S rRNA gene sequences of the confamilial species *M. nigricans* (blue marlin) and *T. angustirostris* (shortbill spearfish) also showed no sequence divergence, a finding confirmed by the zero branch lengths for these species in the NJ tree (Fig. 3).

Conclusions

To our knowledge, this is the first study that has evaluated the utility of partial fragments of the 16S and 12S rRNA genes to serve as universal markers for the identification of a wide variety of fish species, particularly those commonly encountered on the South African market. The sequence data generated in this study, much of which were previously unavailable in GenBank, holds value for future genetic studies on fish species and may also prove useful in the development of alternative high-throughput identification technologies, such as multi-species DNA microarrays. Indeed, this work represents an important step forward in the ability to identify fish species in South Africa at the molecular level, providing the necessary genetic information to permit the identification of at least 48 of 53 (*ca.* 91%) fish to the level of genus and at least 38 of 53 (72%) to the species level. Nonetheless, the results obtained here indicate that the investigated mitochondrial DNA markers most likely do not contain sufficient nucleotide variation to explicitly differentiate certain closely-related congeneric fish species. In the light of these findings, it is recommended that partial 16S and 12S rRNA gene sequences may be useful for complimenting identification results based on alternative DNA markers, however, it does not seem likely that either could be used alone to achieve unambiguous species resolutions for a large number of commercial fish species in South Africa. One or more faster-evolving genes, such as the COI or cyt *b* regions, may be more useful in differentiating closely-related fish species and, as such, could be more suited to serve as universal markers for fish identification purposes.

References

Akimoto, S., Kinoshita, S., Sezaki, K., Mitani, I. & Watabe, S. (2002). Identification of alfonsino and related fish species belonging to the genus *Beryx* with mitochondrial 16S rRNA gene and its application on their pelagic eggs. *Fisheries Science*, **68**, 1242-1249.

Ardura, A., Linde, A.R., Moreira, J.C. & Garcia-Vazquez, E. (2010). DNA barcoding for conservation and management of Amazonian commercial fish. *Biological Conservation*, **143**, 1438-1443.

Balitzki-Korte, B., Anslinger, K., Bartsch, C. & Rolf B. (2005). Species identification by means of pyrosequencing the mitochondrial 12S rRNA gene. *International Journal of Legal Medicine*, **119**, 291-294.

Bartlett, S.E. & Davidson, W.S. (1992). FINS forensically informative nucleotide sequencing – a procedure for identifying the animal origin of biological specimens. *Biotechniques*, **12**, 408-411.

Buyck, B. (1999). Taxonomists are an endangered species in Europe. *Nature*, **401**, 321.

Cawthorn, D.M., Steinman, H.A. & Witthuhn, R.C. (2011). Evaluating the availability of fish species on the South African market and the factors undermining sustainability and consumer choice. *Food Control*, **22**, 1748-1759.

Céspedes, A., García, T., Carrera, E., González, I., Sanz, B., Hernández, P.E. & Martín, R. (1998). Identification of flatfish species using polymerase chain reaction (PCR) amplification and restriction analysis of the cytochrome *b* gene. *Journal of Food Science*, **63**, 206-209.

Chauhan, T. & Rajiv, K. (2010). Molecular markers and their applications in fisheries and aquaculture. *Advances in Bioscience and Biotechnology*, **1**, 281-291.

Comesaña, A.S., Abella, P. & Sanjuán, A. (2003). Molecular identification of five commercial flatfish species by PCR–RFLP analysis of a 12S rRNA gene fragment. *Journal of the Science of Food and Agriculture*, **83**, 752-759.

Comi, G., Iacumin, L., Rantsiou, K., Cantoni, C. & Cocolin, L. (2005). Molecular methods for the differentiation of species used in production of cod-fish can detect commercial frauds. *Food Control*, **16**, 37-42.

Di Finizio, A., Guerriero, G., Russo, G.L. & Ciarcia, G. (2007). Identification of gadoid species (Pisces, Gadidae) by sequencing and PCR–RFLP analysis of mitochondrial 12S and 16S rRNA gene fragments. *European Food Research and Technology*, **225**, 337-344.

FAO (Food and Agriculture Organization of the United Nations) (2009). *The State of World Fisheries and Aquaculture 2008*. Pp. 1-175. Rome, Italy: FAO.

Felsenstein J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783-791.

Gharrett, A.J., Gray, A.K. & Heifetz, J. (2001). Identification of rockfish (*Sebastes* spp.) by restriction site analysis of the mitochondrial ND-3/ND-4 and 12S/16S rRNA gene regions. *Fishery Bulletin*, **99**, 49-62.

Girish, P.S., Anjaneyulu, A.S.R., Viswas, K.N., Anand, M., Rajkumar, N., Shivakumar, B.M. & Bhaskar, S. (2004). Sequence analysis of mitochondrial 12S rRNA gene can identify meat species. *Meat Science*, **66**, 551-556.

Hajibabaei, M., Singer, G.A.C., Hebert, P.D.N. & Hickey, D.A. (2007). DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends in Genetics*, **23**, 167-172.

Hebert, P.D.N, Ratnasingham, S. & deWaard, J.R. (2003). Barcoding animal life: cytochrome *c* oxidase subunit I divergences among closely related species. *Proceedings of the Royal Society B*, **270**, S96-S99.

Infante, C., Crespo, A., Zuasti, E., Ponce, M., Pérez, L., Funes, V., Catanese, G. & Manchado, M. (2006). PCR-based methodology for the authentication of the Atlantic mackerel *Scomber scombrus* in commercial canned products. *Food Research International*, **39**, 1023-1028.

INFOSA (2007). Fisheries in the SADC region: selected country profiles. [Internet document]. URL www.infosa.org.na/dloads/restrict/country%20profile/Fisheries.-pdf. 23 August 2010.

Ishizaki, S., Yokoyama, Y., Oshiro, N., Teruya, N., Nagashima, Y., Shiomi, K. & Watabe S. (2006). Molecular identification of pufferfish species using PCR amplification and restriction analysis of a segment of the 16S rRNA gene. *Comparative Biochemistry and Physiology - Part D*: *Genomics and Proteomics*, **1**, 139-144.

Jacquet, J.L. & Pauly, D. (2008). Trade secrets: renaming and mislabelling of seafood. *Marine Policy*, **32**, 309-318.

Karlsson, A.O. & Holmlund, G. (2007). Identification of mammal species using species-specific DNA pyrosequencing. *Forensic Science International*, **173**, 16-20.

Kimura, M. (1980). A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**, 111-120.

Kitano, T., Umetsu, K., Tian, W. & Osawa, M. (2007). Two universal primer sets for species identification among vertebrates. *International Journal of Legal Medicine*, **121**, 423-427.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S., Paabom, S., Villablanca, F.X. & Wilson, A.C. (1989). Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences*, **66**, 6196-6200.

Kochzius, M., Seidel, C., Antoniou, A., Botla, S.K., Campo, D., Cariani, A., Garcia-Vazquez, E., Hauschild, J., Hervet, C., Hjörleifsdottir, S., Hreggvidsson, G., Kappel, K., Landi, M., Magoulas, A., Marteinsson, V., Nölte, M., Planes, S., Tinti, F., Turan, C., Venugopal, M.N., Weber, H. & Blohm, D. (2010). Identifying fishes through DNA barcodes and microarrays. *PLoS One*, **5**, e12620.

Lakra, W.S., Verma, M.S., Goswani, M., Lal, K.K., Mohindra, V., Punia, P., Gopalakrishnan, A., Singh, K.V., Ward, R.D. & Hebert, P. (2011). DNA barcoding Indian marine fishes. *Molecular Ecology Resources*, **11**, 60-71.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J. & Higgins, D.G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, **23**, 2947-2948.

Linacre, A. & Tobe, S.S. (2011). An overview to the investigative approach to species testing in wildlife forensic science. *Investigative Genetics*, **2**, 1-9.

Lleonart, J., Taconet, M. & Lamboeuf, M. (2006). Integrating information on marine species identification for fishery purposes. *Marine Ecology Progress Series*, **316**, 231-238.

Lockley, A.K. & Bardsley, R.G. (2000). DNA-based methods for food authentication. *Trends in Food Science and Technology*, **11**, 67-77.

Melton, T. & Holland, C. (2007). Routine forensic use of the mitochondrial 12S ribosomal RNA gene for species identification. *Journal of Forensic Sciences*, **52**, 1305-1307.

Meyer, C.P. & Paulay, G. (2005). DNA barcoding: error rates based on comprehensive sampling. *PLoS Biology*, **3**, e422.

Mitani, T., Akane, A., Tokiyasu, T., Yoshimura, S., Okii, Y. & Yoshida, M. (2009). Identification of animal species using the partial sequences in the mitochondrial 16S rRNA gene. *Legal Medicine*, **11**, S449-S450.

Ogden, R. (2008). Fisheries forensics: the use of DNA tools for improving compliance, traceability and enforcement in the fishing industry. *Fish and Fisheries*, **9**, 462-472.

Parson, W., Pegoraro, K., Niederstätter, H., Föger, M. & Steinlechner, M. (2000). Species identification by means of the cytochrome *b* gene. *International Journal of Legal Medicine*, **114**, 23-28.

Palumbi, S.R. (1996). Nucleic acids II: the polymerase chain reaction. In: *Molecular Systematics* (edited by D.M. Hillis, C. Moritz & B.K. Mable). Pp. 205-247. Massachusetts, USA: Sinauer & Associates Inc.

Pauly, D., Watson, R. & Alder, J. (2005). Global trends in world fisheries: impacts on marine ecosystems and food security. *Philosophical Transactions of the Royal Society B*, **360**, 5-12.

Prakash, P.S., Ghumatkar, M.S., Nandode, S.V., Yogesh, S.K. & Shouche, Y.S. (2000). Mitochondrial 12S rRNA sequence analysis in wildlife forensics. *Current Science*, **78**, 1239-1241.

Rastogi, G., Dharne, M., Bharde, A., Pandav, V.S., Ghumatkar, S.V., Krishnamurthy, R., Patole, M.S. & Shouche, Y.S. (2004). Species determination and authentication of meat samples by mitochondrial 12S rRNA sequence analysis and conformation sensitive gel electrophoresis. *Current Science*, **87**, 1278-1281.

Rastogi, G., Dharne, M.S., Walujkar, S., Kumar, A., Patole, M.S. & Shouche, Y.S. (2007). Species identification and authentication of tissues of animal origin using mitochondrial and nuclear markers. *Meat Science*, **76**, 666-674.

Robin, E.D. & Wong R. (1988). Mitochondrial-DNA molecules and virtual number of mitochondria per cell in mammalian-cells. *Journal of Cell Physiology*, **136**, 507-513.

Rock, J., Costa, F.O., Walker, D.I., North, A.W., Hutchinson, W.F. & Carvalho, G.R. (2008). DNA barcodes of fish of the Scotia Sea, Antarctica indicate priority groups for taxonomic and systematics focus. *Antarctic Science*, **20**, 253-262.

Schander, C. & Willassen, E. (2005). What can biological barcoding do for marine biology? *Marine Biology Research*, **1**, 79-83.

Steinke, D., Zemlak, T.S., Hebert, P.D.N. & Adamowicz, S.A. (2009). DNA barcoding of Pacific Canada's fishes. *Marine Biology*, **156**, 2641-2647.

Su, B., Wang, Y.X., Lan, H., Wang, W. & Zhang, Y. (1999). Phylogenetic study of complete cytochrome *b* genes in musk deer (Genus *Moschus*) using museum samples. *Molecular Phylogenetics and Evolution*, **12**, 241-249.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, **24**, 1596-1599.

Tautz, D., Arctander, P., Minelli, A., Thomas, R.H. & Vogler, A.P. (2003). A plea for DNA taxonomy. *Trends in Ecology & Evolution*, **18**, 70-74.

Tobe, S.S. & Linacre, A.M.T. (2008). A technique for the quantification of human and non-human mammalian mitochondrial DNA copy number in forensic and other mixtures. *Forensic Science International: Genetics*, **2**, 249-256.

Van der Elst, R. (1997). *A Guide to Common Sea Fishes of Southern Africa*. Pp. 7-22. Cape Town, South Africa: Struik Publishers.

Vawter, L. & Brown, W.M. (1986). Nuclear and mitochondrial DNA comparisons reveal extreme rate variation in the molecular clock. *Science*, **234**, 194-196.

Vences, M., Thomas, M., van der Meijden, A., Chiari, Y. & Vieites, D.R. (2005). Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. *Frontiers in Zoology*, **2**, 1-12.

Von der Heyden, S., Barendse, J., Seebregts, A.J. & Matthee, C.A. (2010). Misleading the masses: detection of mislabelled and substituted frozen fish products in South Africa. *ICES Journal of Marine Science*, **67**, 176-185.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R. & Hebert, P.D.N. (2005). DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B*, **360**, 1847-1857.

Ward, R.D., Holmes, B.H., White, W.T. & Last, P.R. (2008). DNA barcoding Australasian chondrichthyans: results and potential uses in conservation. *Marine and Freshwater Research*, **59**, 57-71.

Ward, R.D., Hanner, R. & Herbert, P.D.N. (2009). The campaign to DNA barcode all fishes, FISH-BOL. *Journal of Fish Biology*, **74**, 329-356.

Zhang, J., Huang, H., Cai, Z. & Huang, L. (2007). Species identification in salted products of red snappers by semi-nested PCR–RFLP based on the mitochondria 12S rRNA gene sequence. *Food Control*, **18**, 1331-1336.

CHAPTER 6

ESTABLISHMENT OF A MITOCHONDRIAL DNA SEQUENCE DATABASE FOR THE IDENTIFICATION OF FISH SPECIES COMMERCIALLY AVAILABLE IN SOUTH AFRICA

Abstract

The limitations intrinsic to morphology-based identification systems have created an urgent need for reliable genetic methods that enable the unequivocal recognition of fish species, particularly those that are prone to overexploitation and/or market substitution. The aim of this study was to develop a comprehensive reference library of DNA sequence data to allow the explicit identification of 53 commercially available fish species in South Africa, most of which were locally-caught marine species. Sequences of approximately 655 base pairs (bp) were generated for all species from the cytochrome *c* oxidase I (COI) gene, the region widely adopted for DNA barcoding. Specimens of the genus *Thunnus* were examined in further detail, employing additional mitochondrial DNA (mtDNA) control region sequencing. Cumulative analysis of the sequences from the COI region revealed mean conspecific, congeneric and confamilial Kimura two parameter (K2P) distances of 0.10%, 4.58% and 15.43%, respectively. The results showed that the vast majority (98%) of fish species examined could be readily differentiated by their COI barcodes, but that supplementary control region sequencing was more useful for the discrimination of three *Thunnus* species. Additionally, the analysis of COI data raised the prospect that *Thyrsites atun* (snoek) could constitute a species pair. The present study has established the necessary genetic information to permit the unambiguous identification of 53 commonly marketed fish species in South Africa, the applications of which hold a plethora of benefits relating to ecology research, fisheries management and the control of commercial practices.

Cawthorn, D.M., Steinman, H.A. & Witthuhn, R.C. (2011). Establishment of a mitochondrial DNA sequence database for the identification of fish species commercially available in South Africa. *Molecular Ecology Resources*, **11**(6), 979-991.

Introduction

The rapidly declining state of the world's fish stocks and the escalating incidence of fraud and species substitutions on global markets have culminated in an urgent need to reassess the manner in which fish species are identified (Di Finizio *et al*., 2007; Jacquet & Pauly, 2008). Over 30 000 fish species exist worldwide (Froese & Pauly, 2010) and while these can often be discriminated by knowledgeable individuals when in their whole state, morphological differences may be subtle in some cases or not easily recognised when foreign fish are traded over international borders (Teletchea, 2009). Furthermore, when the industrial processing of fish removes the distinguishing external features, identification by means of visual inspection becomes particularly difficult (Gil, 2007).

The sequencing of specific DNA fragments amplified by the polymerase chain reaction (PCR) is considered to be the most accurate and informative method for the identification of fresh, frozen and processed fish species (Lockley & Bardsley, 2000; Gil, 2007; Hubalkova *et al*., 2007). Nonetheless, the success of such authentication techniques relies on the establishment of databases containing reference DNA sequences from a large variety of expertly identified fish specimens (Martinez *et al*., 2005; Steinke *et al*., 2009a; Ward *et al*., 2009). The sequences of unknown samples can then be compared and potentially matched with those sequences from reference specimens that are deposited in international, open-access sequence databases, such as GenBank (www.ncbi.nlm.nih.gov) and the Barcode of Life Database (BOLD) (www.boldsystems.org).

The ability to make unambiguous species identifications using sequencing methods is highly dependent on the specific DNA marker chosen for analysis. For such purposes, it is imperative that the inter-species sequence variation presented in the region of interest is substantially greater than the intra-species variation (Ward *et al*., 2005). Although both the nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) may be targeted for species identification, the analysis of mtDNA offers numerous advantages over the nDNA for fish species identification (Gil, 2007). The maternal inheritance and lack of recombination of vertebrate mtDNA means that it is conserved across extended evolutionary distances, and the high copy number and mutation rate makes it suitable for discriminating between a large number of species (Rokas *et al*., 2003; Teletchea, 2009). In particular, the mitochondrial ribosomal RNA (rRNA) genes, cytochrome *b* gene and the control region have been used in the past for the identification of different fish species and for the detection of commercial substitution of fish products (Lockley & Bardsley, 2000; Rasmussen & Morrisey, 2009).

In order to promote rapid and automated sample processing, it would be beneficial if the sequence diversity in a single standardised gene region could be globally utilised for species identification (Stoeckle, 2003; Blaxter, 2004). Recently it was proposed, and subsequently demonstrated, that an approximately 650 bp region of the mitochondrial COI gene has sufficient discriminatory power to serve as a unique 'barcode' for the identification of the large majority of animal species (Hebert *et al*., 2003a,b; Ward *et al*., 2005; Dawnay *et al*., 2007). The acceptance of the COI gene by the Consortium for the Barcode of Life (CBOL) for DNA barcoding purposes has provoked a number of international collaborative research efforts, including the Fish Barcode of Life Initiative (FISH-BOL), which aims to barcode all of the world's fish species (Ward *et al*., 2009).

South Africa comprises one of the most well-established and lucrative fishing nations on the African continent (FAO, 2010), owing primarily to the extensive variety of fish species that are found within its national fishing zone. In 2007, capture production for fisheries in South Africa was estimated at 670 571 metric tons (live weight), with the bulk of this production being consumed domestically (FAO, 2009). In spite of the commercial importance of the fishing industry in this country and the fact that species substitutions have been suspected on the local market (Smith & Smith, 1966; Atkins, 2010; Von der Heyden *et al.*, 2010), the public availability of reference DNA sequence data for many fish species commonly encountered in South Africa is limited or non-existent. The information that is available is fragmented, representing sequences from varying gene regions for different taxonomic groups. This lack of consolidated genetic data presents a major challenge for the identification of fish species in South Africa using DNA-based methods.

The aim of this study was to construct a mtDNA database for 53 commercially significant fish species in South Africa, containing sufficient sequence data to permit the unambiguous identification of all reference specimens. Fulfillment of this aim entailed the generation of sequence data from two different mtDNA loci (the COI for all fishes, as well as the control region for *Thunnus* species), evaluation of the utility of these DNA markers to allow explicit species resolutions, and the submission of reference sequences to public databases to allow remote comparisons for future fish authentication studies.

Materials and Methods

Samples

Fifty three putative fish species, representing 42 genera within 23 families and 9 orders of ray-finned fishes (*Actinopterygii*), were collected from major trawling companies, fish processing facilities and the Department of Agriculture, Forestry and Fisheries (DAFF) in South Africa. The large majority of the fish in this collection were marine species, 47 of which were caught locally. Seven species were imported from outside African borders, but were included as these are commonly available on the South African market (Cawthorn *et al*., 2011a). At least 3 specimens of each species were analysed, totaling 194 individuals. The numbers (N) of specimens per species ranged from 3 to 12, with a mean of 3.66 specimens per species. All specimens were morphologically identified at the species level by fish taxonomists based on established meristic, morphometric and colouration criteria. Vouchers specimens for all samples are stored at the Department of Food Science, Stellenbosch University, South Africa.

Extraction of DNA

Tissue subsamples were excised from the lateral muscle on the right side of each fish specimen using a sterile scalpel and forceps. DNA was extracted using the SureFood® PREP allergen kit (r-Biopharm, supplied by AEC-Amersham, Cape Town, South Africa) according to the instructions of the manufacturer. This method was found to extract the highest yields of DNA from fish muscle in a previous comparative study (Cawthorn *et al*., 2011b). The concentration and purity of the DNA extracts was estimated at 260 nm and 280 nm in a spectrophotometer (Beckman Coulter DU530, Beckman Instruments, Fullerton, USA). Extracted DNA was stored at -20 °C until further use.

PCR amplification

The extracted DNA from all specimens was used as a template for PCR amplification with oligonucleotide primers targeting the COI gene (Table 1). A 450 bp fragment of the mtDNA control region (Table 1) was also amplified in cases where the identities of closely-related species could not be resolved by COI sequencing. PCR amplifications were carried out in a Mastercycler Personal (Eppendorf, Germany) utilising the reaction mixtures and thermal cycling regimes shown in Table 2. PCR products were resolved by electrophoresis (90 volts, 45 min) in 1.5% (m/v) agarose (Sigma-Aldrich, Gauteng, South Africa) gels and were visualised under an ultraviolet light.

Table 1 PCR primers used for the generation of mitochondrial DNA sequence data for fish species commercially available in South Africa

Primer	Primer sequence (5' to 3')	mtDNA target	Amplicon size (bp)	Reference
Fish DNA barcoding primer cocktail (C_FishF1t1 / C_FishR1t1)				
VF2_t1	TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC	Cytochrome *c* oxidase 1 (COI) gene	652	Ivanova *et al.*, 2007
FishF2_t1	TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC			
FishR2_t1	CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA			
FR1d_t1	CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA			
M13F *	TGTAAAACGACGGCCAGT			Messing, 1983
M13R *	CAGGAAACAGCTATGAC			
Control region primers				
L15998	TAC CCC AAA CTC CCA AAG CTA	Control region (D-loop)	450	Alvarado Bremer, 1994
CSBDH	TgA ATT AGG AAC CAG ATG CCA G			

* Sequencing primers for M13-tailed PCR products

Table 2 Reaction mixtures and thermal cycling parameters used for PCR amplifications

Primers	**Total volume**	**Primer 1** (10 µM)	**Primer 2** (10 µM)	**Primer 3** (10 µM)	**Primer 4** (10 µM)	***Taq* DNA polymerase**[a] (5 U.µl^{-1})	**Reaction buffer**[a] (10 X)	**$MgCl_2$**[a] (25 mM)	**dNTPs**[b] (10 mM)	**DNA template**	**PCR cycling conditions**
C_FishF1t1/ C_FishR1t1	25 µl	0.25 µl (0.10 µM)	0.25 µl (0.10 µM)	0.25 µl (0.10 µM)	0.25 µl (0.10 µM)	0.125 µl (0.625 U)	2.5 µl (1 X)	2.5 µl (2.5 mM)	0.5 µl (0.2 mM)	2.0 µl (*ca*. 2 µg)	94 °C - 2 min; 35 x (94 °C - 30 s, 52 °C - 40 s, 72 °C - 60 s); 72 °C - 10 min
L15998/ CSBDH	25 µl	1.0 µl (0.40 µM)	1.0 µl (0.40 µM)			0.10 µl (0.5 U)	2.5 µl (1 X)	2.0 µl (2.0 mM)	2.0 µl (0.8 mM)	1.0 µl (*ca*. 1 µg)	94 °C - 5 min; 35 x (94 °C - 45 s, 54 °C - 45 s, 72°C - 60 s); 72°C - 10 min

[a]Super-Therm, supplied by Southern Cross Biotechnologies, Cape Town, South Africa
[b]AB gene, supplied by Southern Cross Biotechnologies

Sequencing and sequence analysis

Purification of PCR products was performed using the NucleoFast 96 PCR Clean-up Kit (Macherey-Nagel, supplied by Separations, Gauteng, South Africa) according to the instructions of the manufacturer. The purified PCR products were sequenced using BigDye chemistry and were analysed on an ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, USA). Primers for M13-tailed PCR products (Messing, 1983) (Table 1) were used for the sequencing of COI amplicons, while the PCR amplification primers were used as sequencing primers for the control region amplicons (Table 1). The COI sequences were aligned using the complete alignment application in Clustal X version 2.0 (Larkin *et al*., 2007) and the same procedure was used for the alignment of those sequences generated from the mtDNA control region. All generated DNA sequences were submitted to GenBank. The GenBank accession numbers for COI sequences are given in Table 3, while the accession numbers for the control region sequences generated from three *Thunnus* species are HQ853210 - HQ853219. COI sequences, along with specimen and collection details, were also submitted to BOLD under the project name 'Barcoding marine fish species of South Africa'.

The mean overall nucleotide base frequencies for the COI sequences (Table 4) were computed in BOLD. COI sequence divergence values, hereafter referred to as distance or D, were computed using the Kimura two parameter (K2P) distance model (Kimura, 1980). The mean K2P distances in percentages within different taxonomic levels are provided in Table 5, the distributions of which are represented in Figure 1. The neighbor-joining (NJ) tree based on COI sequences (Fig. 2) was compiled in MEGA version 4.0 (Tamura *et al*., 2007) using the pairwise deletion of missing nucleotide data option and the robustness of topology nodes was evaluated using the non-parametric bootstrap method with 1000 replications (Felsenstein, 1985).

Additional GenBank sequences

The validity of COI and control region sequencing for *Thunnus* species identification was further investigated by supplementing the sequences generated in this study for South African *Thunnus* specimens with additional sequences from GenBank for all eight recognised species within this genus (Collette *et al*., 2001). The number of sequences acquired per species was based on their availability in GenBank. For the COI gene, 38 *Thunnus* sequences were analysed in total. These included six sequences from *Thunnus* *alalunga* (albacore), six from *Thunnus obesus* (bigeye tuna), five sequences each from *Thunnus albacares* (yellowfin tuna), *Thunnus orientalis* (northern bluefin tuna), *Thunnus maccoyii* (southern bluefin tuna) and *Thunnus atlanticus* (Atlantic blackfin tuna), as well as four from *Thunnus tonggol* (longtail tuna). COI data for *Thunnus thynnus* (Atlantic bluefin tuna) were limited in GenBank and only two sequences from this species were included. The geographical origins and accession numbers of all investigated COI sequences are provided in Figure 3.

For the mtDNA control region, 27 sequences from the eight members of the genus *Thunnus* were analysed, which included six sequences each from *T. obesus* and *T. albacares*, five from *T. alalunga*, four from *T. thynnus* and three from *T. orientalis*. The single control region sequences available in GenBank for *T. atlanticus*, *T. maccoyii* and *T. tonggol* were also included. The accession numbers of all evaluated control region sequences of the *Thunnus* species are provided in Figure 4. K2P distances and NJ trees for the COI and control region sequences of *Thunnus* species were computed in MEGA 4.

Results and discussion

Common names, species and family details, as well as GenBank accession numbers for all fish specimens sequenced for the COI gene region are presented in Table 3. The DNA obtained from all 53 examined fish species was readily amplified using the barcoding primer cocktail described by Ivanova *et al*. (2007) (Table 1), generating a total of 194 sequences owing to the analysis of multiple specimens of each species. Mitochondrial control region amplicons were also successfully recovered from ten specimens representing three species of the genus *Thunnus*. New DNA sequence data were generated in this study for many commercially significant fish species in South Africa that were previously unavailable in GenBank. For 23% of the fish species evaluated, this was the first time that DNA sequence data of any kind were submitted to GenBank. COI sequences were deposited in GenBank for the first time for 30% of the species (Table 3).

Cytochrome *c* oxidase I (barcoding) sequences

Read lengths of the 194 COI sequences were approximately 655 bp, with less than 2% of these being less than 600 bp in length. No indels, stop codons or sequences indicative of NUMTs (nuclear DNA sequences originating from mitochondrial DNA sequences) were

Table 3 Species, sample sizes (N), localities and GenBank accession numbers of all specimens analysed in this study, with grey shading representing the first entries into GenBank for the given gene region

Order	Family	Species	Common name * (Local name)	N	Country of origin	COI gene GenBank accession No.
Clupeiformes	Clupeidae	*Clupea harengus*	Atlantic herring	3	North sea	HQ611120 - HQ611122
		Sardinops sagax	South American pilchard (pilchard/sardine)	3	South Africa	HQ611132 - HQ611134
	Engraulidae	*Engraulis encrasicolus*	European anchovy (anchovy)	3	South Africa	HM007778 - HM007780
		Engraulis japonicus	Japanese anchovy (Cape anchovy)	3	South Africa	HM007796 - HM007798
Gadiformes	Merlucciidae	*Merluccius capensis*	Shallow-water Cape hake	4	South Africa	HM007690 - HM007692, HQ611082
		Merluccius paradoxus	Deep-water Cape hake	7	South Africa	HM007683 - HM007689
Lophiiformes	Lophiidae	*Lophius vomerinus*	Devil anglerfish (Cape monk)	3	South Africa	HM007765 - HM007767
Ophidiiformes	Ophidiidae	*Genypterus blacodes*	Pink cusk-eel (ling)	3	New Zealand / Argentina	HQ611135, HQ611136, HQ611137
		Genypterus capensis	Kingklip	12	South Africa	HM007735 - HM007746
Perciformes	Bramidae	*Brama brama*	Atlantic pomfret (angelfish)	4	South Africa	HQ611083 - HQ611086
	Carangidae	*Seriola lalandi*	Yellowtail amberjack	4	South Africa	HM007727 - HM007730
		Seriola quinqueradiata	Japanese amberjack	3	China	HQ641665 - HQ641667
		Trachurus capensis	Cape horse mackerel (maasbanker)	3	South Africa	HM007775 - HM007777
	Coryphaenidae	*Coryphaena hippurus*	Common dolphinfish (dorado)	4	South Africa	HM007704 - HM007707
	Centrolophidae	*Centrolophus niger*	Rudderfish (black ruff)	3	South Africa	HM007793 - HM007795
		Schedophilus velaini	Violet warehou (black butterfish)	3	South Africa	HQ611129 - HQ611131
		Seriolella brama	Common warehou	3	Australia / New Zealand	HM007731 - HM007733, HM007734

Table 3 (continued)

Order	Family	Species	Common name * (Local name)	N	Country of origin	COI gene GenBank accession No.
Perciformes	Epigonidae	*Epigonus telescopus*	Black cardinal fish (cardinal)	3	South Africa	HM007701 - HM007703
	Gempylidae	*Lepidocybium flavobrunneum*	Escolar (butterfish)	3	South Africa	HM007724 - HM007726
		Ruvettus pretiosus	Oilfish (butterfish)	3	South Africa	HM007721 - HM007723
	Gempylidae	*Thyrsites atun*	Snoek	6	South Africa / New zealand	HQ611106 - HQ611108, HQ611109, HQ611110, HQ641670
	Istiophoridae	*Makaira nigricans*	Blue marlin	3	South Africa	HQ611114 - HQ611116
		Tetrapturus angustirostris	Shortbill spearfish	3	South Africa	HQ611111 - HQ611113
	Sciaenidae	*Argyrosomus inodorus*	Mild meagre (silver kob)	7	South Africa	HM007711 - HM007717
		Argyrosomus japonicus	Japanese meagre (dusky kob)	3	South Africa	HM007718 - HM007720
		Atractoscion aequidens	Geelbek croaker (geelbek/Cape salmon)	5	South Africa	HM007696 - HM007700
	Scombridae	*Gasterochisma melampus*	Butterfly kingfish (gastora)	3	South Africa	HM007708 - HM007710
		Katsuwonus pelamis	Skipjack tuna	3	South Africa	HQ611090 - HQ611092
		Scomber japonicus	Chub mackerel	3	South Africa	HQ611117 - HQ611119
		Scomberomorus commerson	Spanish mackerel (king mackerel/couta)	3	South Africa	HM007790 - HM007792
		Thunnus alalunga	Albacore (longfin tuna)	3	South Africa	HM007772 - HM007774
		Thunnus albacares	Yellowfin tuna	4	South Africa	HM007768 - HM007771
		Thunnus obesus	Bigeye tuna	3	South Africa	HQ611138 - HQ611140
	Serranidae	*Cephalopholis sonnerati*	Tomato hind (tomato rockcod)	3	South Africa	HQ611096 - HQ611098

Table 3 (continued)

Order	Family	Species	Common name * (Local name)	N	Country of origin	COI gene GenBank accession No.
Perciformes	Serranidae	*Epinephelus marginatus*	Dusky gouper (yellowbelly rockcod)	3	South Africa	HQ611093 - HQ611095
	Sparidae	*Argyrozona argyrozona*	Carpenter seabream (silverfish)	3	South Africa	HM007753 - HM007755
		Cheimerius nufar	Santer seabream	4	South Africa	HQ611102 - HQ611105
		Chrysoblephus anglicus	Englishman seabream	3	South Africa	HQ611099 - HQ611101
		Chrysoblephus laticeps	Roman seabream	3	South Africa	HM007750 - HM007752
		Chrysoblephus puniceus	Slinger seabream	3	South Africa	HQ611087 - HQ611089
		Pachymetopon blochii	Hottentot seabream	3	South Africa	HM007693 - HM007695
		Pterogymnus laniarius	Panga seabream	3	South Africa	HM007781 - HM007783
		Rhabdosargus globiceps	White stumpnose	3	South Africa	HM007759 - HM007761
Perciformes	Trichiuridae	*Lepidopus caudatus*	Silver scabbardfish (buttersnoek)	3	South Africa	HM007784 - HM007786
	Xiphiidae	*Xiphias gladius*	Swordfish	3	South Africa	HM007787 - HM007789
Pleuronectiformes	Soleidae	*Austroglossus microlepis*	West coast sole	6	South Africa	HM007674 - HM007678, HQ641664
		Austroglossus pectoralis	Mud sole (East coast sole)	4	South Africa	HM007679 - HM007682
Salmoniformes	Salmonidae	*Oncorhynchus mykiss*	Rainbow trout	3	South Africa	HQ611123 - HQ611125
		Oncorhynchus keta	Chum salmon (Alaskan salmon)	3	USA	HQ611126 - HQ611128
		Salmo salar	Atlantic salmon	5	Norway / Canada	HM007799 - HM007801, HQ641668 - HQ641669
Scorpaeniformes	Sebastidae	*Helicolenus dactylopterus*	Blackbelly rosefish (jacopever)	3	South Africa	HM007747 - HM007749
	Triglidae	*Chelidonichthys capensis*	Cape gurnard	3	South Africa	HM007756 - HM007758
Zeiformes	Zeidae	*Zeus capensis*	Cape dory	3	South Africa	HM007762 - HM007764

* Common names are from FishBase (www.fishbase.org)

encountered in any of the COI sequences, a result in agreement with previous fish barcoding reports (Steinke *et al*., 2009b; Lakra *et al*., 2011).

The mean overall nucleotide base frequencies observed for the COI sequences from the 53 fish species (Table 4) corresponded with those COI nucleotide frequencies reported by Ward *et al*. (2005) for 143 Australian teleost species. Overall, most nucleotide changes were observed at the third codon base position, as illustrated by the standard errors of the GC percentages of 0.40, 0.11 and 0.03 at the third, first and second base positions, respectively (Table 4). Such a finding is concurrent with the fact that in protein-coding DNA regions, the degenerate nature of the genetic code results in the third codon base evolving the most rapidly, with most of these changes being synonymous and not leading to amino acid changes. It is reportedly the third base variability that affords COI barcoding with its power to discriminate closely-related species (Ward & Holmes, 2007).

Although the primary aim of DNA barcoding is to delineate species boundaries and to establish identification systems, phylogenetic relationships were apparent in the COI sequence data. Species within genera consistently clustered together, as did genera within families (Fig. 2). As anticipated, the mean K2P distances within species, genera and families increased with taxonomic rank (Table 5, Fig. 1). The mean distance between genera within families (D = 15.50%) was more than 3-fold greater than that seen within species of genera (4.58%). In turn, the mean distance between species within genera was *ca*. 45-fold greater than the mean distance between individuals within species (D = 0.10%), demonstrating the general discriminative ability of COI barcoding.

The average within-species distance observed for the COI region in this study (D = 0.10%) was somewhat lower than the corresponding values of 0.25% and 0.30% reported in other barcoding studies on marine fish (Steinke *et al*., 2009a; Lakra *et al*., 2011). Previous studies on several hundred marine fish species (Ward *et al*., 2005; 2008; Rock *et al*., 2008; Steinke *et al*., 2009a; 2009b) revealed that more than 95% of intra-species comparisons showed an average COI divergence of less than 2%. In this study, all (100%) of the intra-species values showed less than 2% divergence, with 97% of these exhibiting divergence values below 1% (98% of species within the study) (Fig. 1). Zero intra-species sequence divergence was found for 45 of 53 (85%) fish species examined, and these 45 species all clustered with 100% bootstrap support in the K2P/NJ tree based on the COI sequences (Fig. 2). Nonetheless, since sample sizes were generally small, further sampling could uncover some intra-specific variability for these species.

Table 4 Mean percentage base compositions (with standard errors) of COI gene sequences

Gene	Number of species	G%	C%	A%	T%	GC%	GC% Codon position 1	GC% Codon position 2	GC% Codon position 3
COI	53	18.63 ± 0.07	28.37 ± 0.12	23.43 ± 0.08	29.58 ± 0.11	47.00 ± 0.15	56.24 ± 0.11	42.87 ± 0.03	41.60 ± 0.40

Table 5 Summary of genetic divergences in the COI region calculated for different taxonomic levels using K2P distances (%). Data are represented for 53 fish species within 42 genera and 23 families

Gene	Comparisons within	No. of sequences	Taxa	No. of comparisons	Minimum distance (%)	Mean distance (%)	Maximum distance (%)	Standard error distance (%)
COI	Species	194	53	320	0.00	0.10	1.54	0.020
	Genus	194	42	199	0.46	4.58	11.47	0.216
	Family	194	23	626	3.33	15.50	23.06	0.139
	Order	194	9	6705	14.49	22.75	29.89	0.033
	Class	194	1	10871	16.06	23.86	30.87	0.020

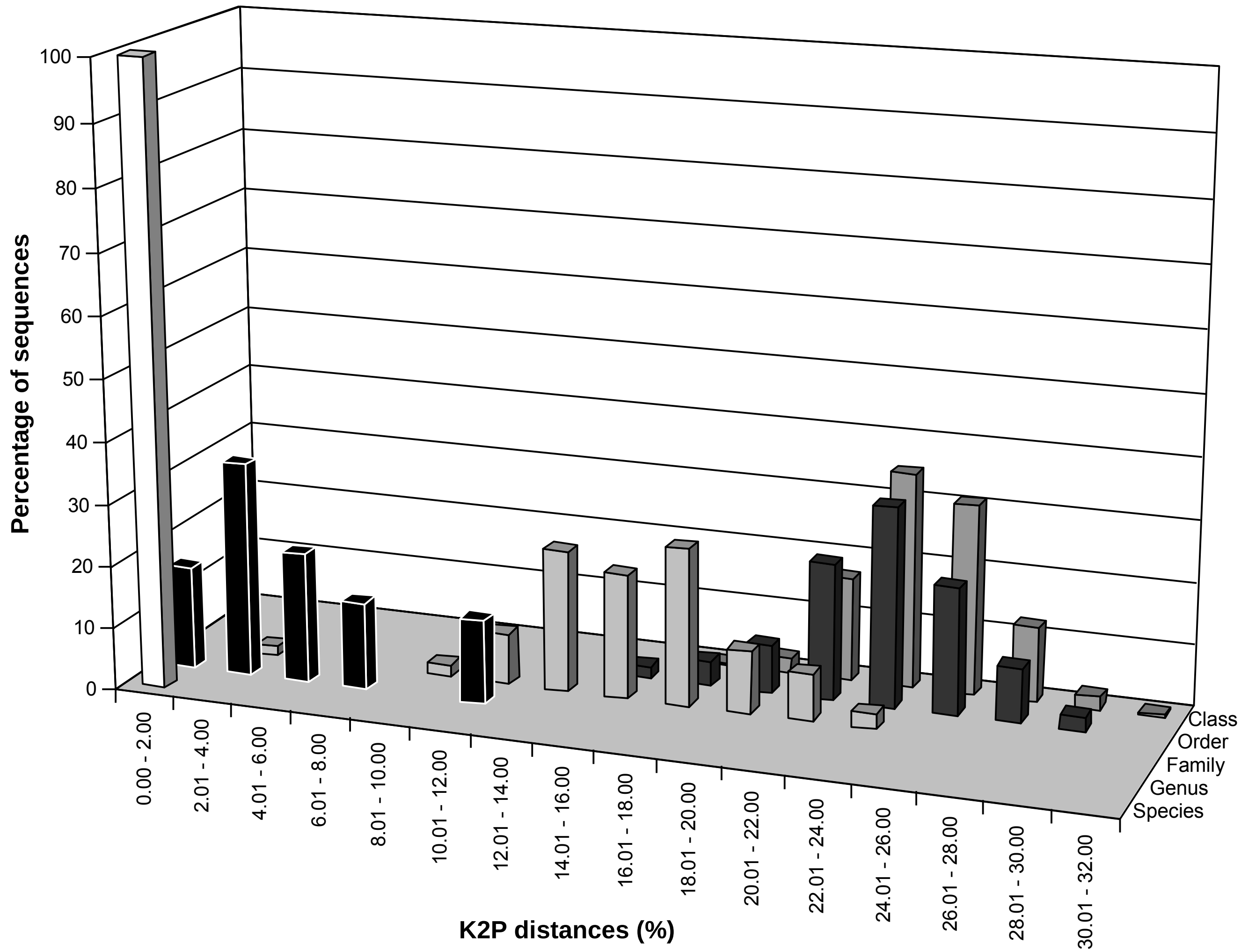
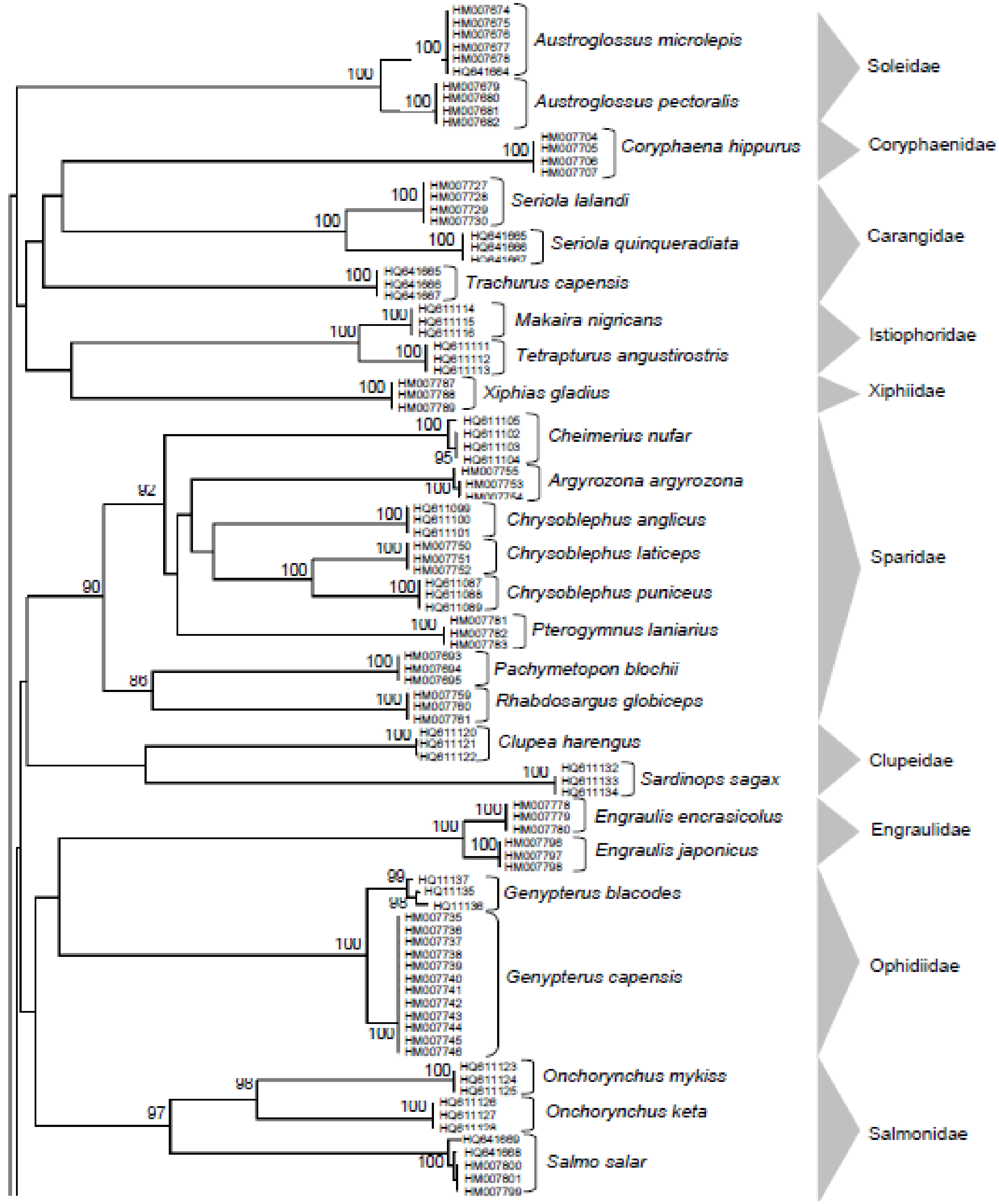


Figure 1 The distribution of K2P distances (in percentages) for the COI region within various taxonomic levels


Austroglossus microlepis
Austroglossus pectoralis
Soleidae
Coryphaena hippurus
Coryphaenidae
Seriola lalandi
Seriola quinqueradiata
Trachurus capensis
Carangidae
Makaira nigricans
Tetrapturus angustirostris
Istiophoridae
Xiphias gladius
Xiphiidae
Cheimerius nufar
Argyrozona argyrozona
Chrysoblephus anglicus
Chrysoblephus laticeps
Chrysoblephus puniceus
Pterogymnus laniarius
Pachymetopon blochii
Rhabdosargus globiceps
Sparidae
Clupea harengus
Sardinops sagax
Clupeidae
Engraulis encrasicolus
Engraulis japonicus
Engraulidae
Genypterus blacodes
Genypterus capensis
Ophidiidae
Onchorynchus mykiss
Onchorynchus keta
Salmo salar
Salmonidae

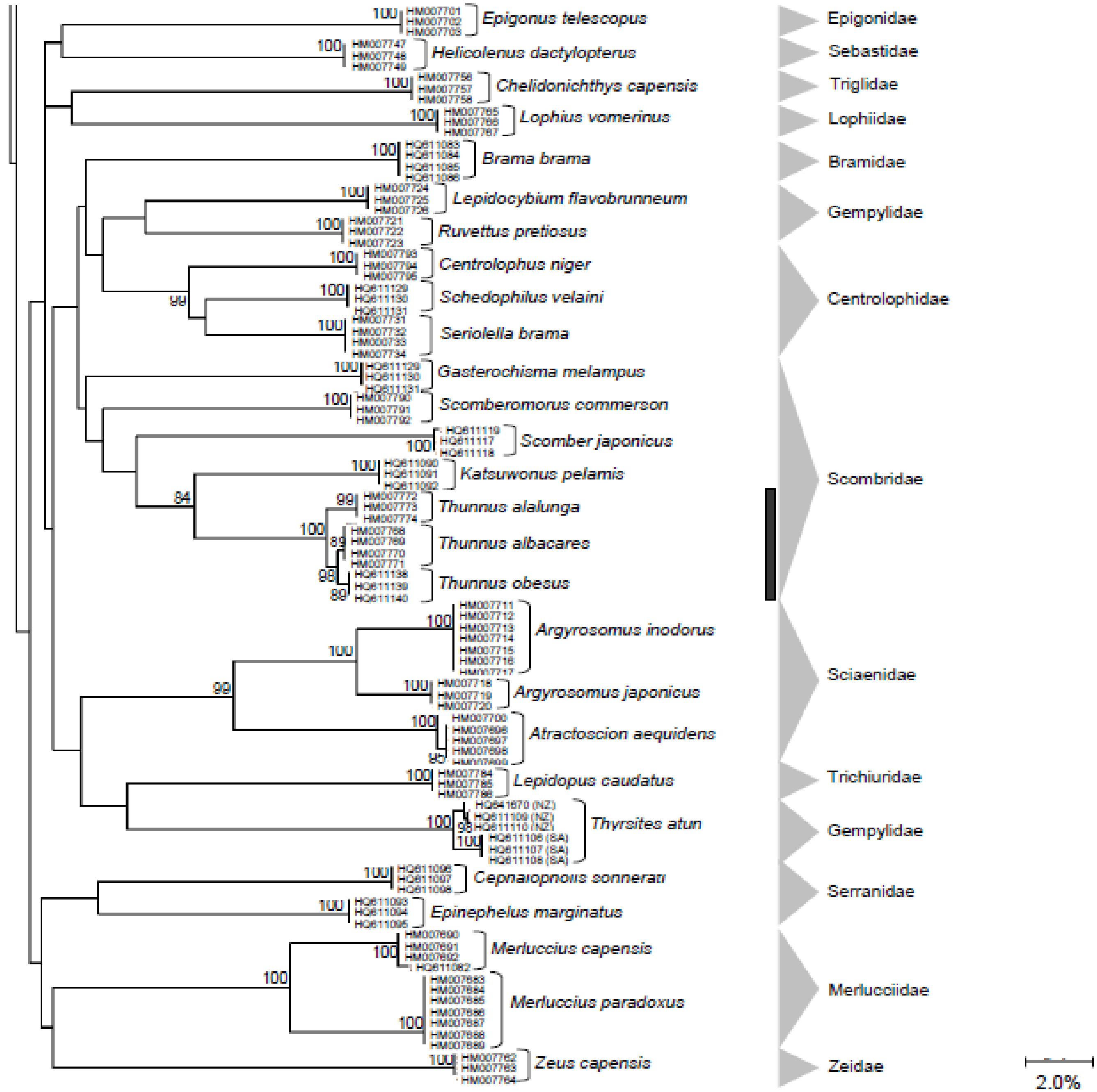


Figure 2 K2P distance neighbour-joining tree of 194 COI sequences from 53 fish species, with GenBank accession numbers for each specimen. Numbers at nodes indicate bootstrap values (values higher than 80 are given). Species showing less than 2% COI divergence are indicated by a black bar.

The maximum intra-species COI variation was observed between specimens of *Thyrsites atun* (snoek), which was one of few species for which both locally caught and imported specimens were analysed. Clear geographic differentiation was apparent for *T. atun*, with one clade represented by individuals from South Africa (SA) and another from New Zealand (NZ), and no shared haplotypes between the SA and NZ specimens. The extent of SA-NZ differentiation, an average K2P distance of 1.54% (Table 5), far exceeded that found within the SA (0.00%) or NZ (0.30%) groups. The three SA *T. atun* specimens separated at a 100% bootstrap value from the three NZ specimens (Fig. 2). Hebert *et al.* (2004) have proposed that genetically divergent specimens may be flagged as provisional species when they show a 10-fold greater mean intra-specific variation than the group under study. It is thus possible that the locally caught *T. atun* and those imported from NZ could potentially refer to two distinct biological species. However, such a distinction would require further clarification, employing extensive morphological and meristic investigations, as well as additional genetic analyses.

For species within genera, the mean COI distance of 4.58% in this study was comparable with that of 3.75% recorded among congeneric fish species from Canadian Pacific waters (Steinke *et al*., 2009a) and that of 6.67% for fish species of the Scotia sea (Rock *et al*., 2008), but was considerably lower than the 9.93% recorded for Australian marine fish (Ward *et al*., 2005). For 50 of 53 (94%) fish species in the dataset, inter-species distance values exceeding 2% were calculated, suggesting that these could be readily discriminated by their COI barcodes. The remaining three species analysed, all members of the genus *Thunnus*, exhibited congeneric distances below 2%. Examination of the NJ tree (Fig. 2) revealed that, although each of three examined *Thunnus* species clustered into a separate grouping with no specimens being misplaced, the genetic distances between the species were small. The mean congeneric distance between specimens of *T. alalunga* and *T. albacares* was 1.51%, and between *T. alalunga* and *T. obseus* was 1.53%. Overall, the minimum inter-species distance (0.46%) was found to be between individuals of *T. albacares* and *T. obesus*. Thus, while DNA barcoding was shown to be a highly effective tool for the identification of the majority of the fish species analysed, these results indicate that the explicit identification of *Thunnus* species using COI sequencing may present considerable challenges.

***Thunnus* species identification**

Members of the genus *Thunnus*, commonly referred to as tunas, are large, migratory fish belonging to the family Scombridae. Although the genus is believed to be monophyletic, the phylogenetic relationships between the individual species within the genus remains controversial and classifications based on morphology, mtDNA and nDNA have not been entirely concordant (Chow & Kishino, 1995; Elliot & Ward, 1995; Alvarado Bremer *et al*., 1997; Lowenstein *et al*., 2009). Of the eight *Thunnus* species recognised by Collette *et al*. (2001), five of these are found in South African waters: *T. obesus*, *T. albacares*, *T. alalunga*, *T. maccoyii* and *T. thynnus* (Froese & Pauly, 2010). Certain members of this genus are extremely commercially significant, which makes them prone to both overexploitation (Safina, 2001; Allen *et al*., 2010) and fraudulent trading practices (FSA, 2000; Jacquet & Pauly, 2008; Lowenstein *et al*., 2009). Since tunas are often presented on the market in processed forms, the development of reliable genetic methodologies is critical for accurate species identifications. Nonetheless, Viñas and Tudela (2009) reported that a number of *Thunnus* species are genetically very similar and that confounded identifications may result if DNA markers with low genetic variation are employed.

DNA barcoding has been proposed as a single universal tool for animal species identifications (Dasmahapatra & Malletv, 2006) and a recent study examining the molecular evolutionary behavior of the COI region in fishes implied that barcoding should be applicable for the identification of all marine species (Ward & Holmes, 2007). However, it has increasingly been recognised that recently-speciated taxa could pose difficulties for identification by DNA barcoding (Elias *et al*., 2007; Lowenstein *et al*., 2009). Since the genus *Thunnus* is recently derived, believed to have evolved during periods of rapid speciation, the analysis of faster-evolving genes rather than slowly-evolving ones is preferable for the identification of these species, since the latter generally do not show sufficient mutations to allow clear distinction between the different members of the genus (Collette *et al*., 2001). While Lowenstein *et al*. (2009) showed that a character-based analysis of COI sequences allowed the discrimination of members of the *Thunnus* genus, reports by the same authors, as well as by Wong and Hanner (2008), have suggested that the genetic-distance methods employed by BOLD and the BLASTn algorithm in GenBank may not permit the clear assignment of *Thunnus* specimens to the species level. Indeed, the preliminary results from the barcoding of three *Thunnus* species in this study (*T. alalunga*, *T. albacares* and *T. obesus*) showed that the unambiguous identification of members of this genus may be problematic based on the COI region. Comparison of the generated COI sequences for these *Thunnus* specimens with those sequences already available in GenBank and in BOLD provided additional support for this finding, where in all cases the sequences of putative species showed equal percentage similarities with those of one or more congeneric species. In order to further investigate the utility of COI barcoding for the unambiguous identification of *Thunnus* species, the generated COI sequences for these three *Thunnus* species were consequently analysed together with representative sequences from GenBank for all eight members of the genus. Figure 3 shows the resulting COI phenogram for 38 specimens, along with the GenBank accession number and geographical origin of each specimen.

Genetic differences within species of *Thunnus* were small at the COI level, with a mean K2P distance of 0.11% (Fig. 3). While members of the same *Thunnus* species clustered under the same nodes, the bootstrap percentage values for most of the species separations were relatively low (60 - 70% level). The mean COI distance between *Thunnus* species was 1.09%, a value more than four times smaller than that found for all 53 fish species analysed in this study (D = 4.58%). *Thunnus alalunga* and *T. orientalis*, the only clade for which high bootstrap support (98%) was obtained in Figure 3, showed the minimum inter-species distance (D = 0.20%) within the *Thunnus* genus. Only a single nucleotide variation was observed in the COI sequences of the two aforementioned species. Interestingly, the sequence of one member of *T. orientalis* (GenBank accession number DQ107592) also differed by a single nucleotide from the sequences of the other members of the species, indicating the problems inherent with making identifications based on single base polymorphisms. Observation of the COI K2P/NJ tree (Fig. 3) revealed that the three species comprising the *Neothunnus* subgenus (*T. albacares*, *T. atlanticus* and *T. tonggol*) (Collette, 1978) assembled into a loosely defined clade with 54% bootstrap support. This finding is in contrast to that of Viñas and Tudela (2009), who reported that members of *Neothunnus* did not group together based on their COI sequences. The mean congeneric distance found between members of *Neothunnus* (D = 0.47%) appears to indicate that these species show very close COI sequence congruence. In particular, the COI sequences of *T. tonggol* differed by only two nucleotides from those of *T. albacares* and by only three nucleotides from those of *T. atlanticus*.

Fundamental to the success of DNA barcoding for species-level identifications is the requirement for members within a species to show substantially less COI variation than that variation seen among different species (Ward *et al*., 2005). This, however, was not found to be the case for all of the examined *Thunnus* species. In fact, the maximum intra-species COI variation seen among *T. maccoyii* specimens (0.46%) was more than 3-fold higher than the minimum inter-species variation (0.15%) between certain *T. alalunga* and *T. orientalis* specimens. The maximum intra-species variation (0.46%) was also higher than the mean inter-species distance between *T. albacares and T. tonggol* (0.38%). Overall, this study has highlighted that *Thunnus* species are likely to pose a challenge for the barcoding system due to the low COI sequence divergence amongst certain congeners and the use of the COI region as a single genetic marker for *Thunnus* species identification has been brought into question.

It has been suggested that a more variable gene region, such as the mtDNA control region, could hold promise for confirming the identity of the closely-related members belonging to the *Thunnus* genus (Viñas & Tudela, 2009). Thus, sequences of the 5' region of the control region were generated for the specimens of *T. alalunga*, *T. albacares* and *T. obesus* examined in this study (accession numbers HQ853210 - HQ853212, HQ853213 - HQ853216, and HQ853217 - HQ853219, respectively). The average read length of the control region sequences was 398 bp. The mean K2P distance found between members of the aforementioned three *Thunnus* species was 12.22%, while the mean within-species distance was 0.18%. The discriminatory power of the control region for *Thunnus* species identification was further validated by comparing the generated sequences with additional sequences from GenBank for all eight *Thunnus* species, the resulting K2P/NJ tree for which is presented in Figure 4.

While it is accepted that the samples sizes of sequences for certain species were small due to their limited availability in GenBank, the control region phenogram (Fig. 4) appears to be considerably more consistent than the corresponding one based on the COI region (Fig. 3), with species separating into distinct clusters with high bootstrap support (80 - 100%). The mean K2P distance between the eight *Thunnus* species (D = 13.20%) was *ca.* 9-fold higher than the within-species value (D = 1.42 %) based on the control region sequences. Both of the aforementioned values were also more than 10-fold greater than the corresponding values found for the eight species based on the COI region, emphasising the greater nucleotide diversity exhibited in the control region of *Thunnus* species. *Thunnus alalunga* and *T. orientalis*, the two species found to differ by only one nucleotide in their COI sequences (D = 0.20%), were easily identifiable by their control

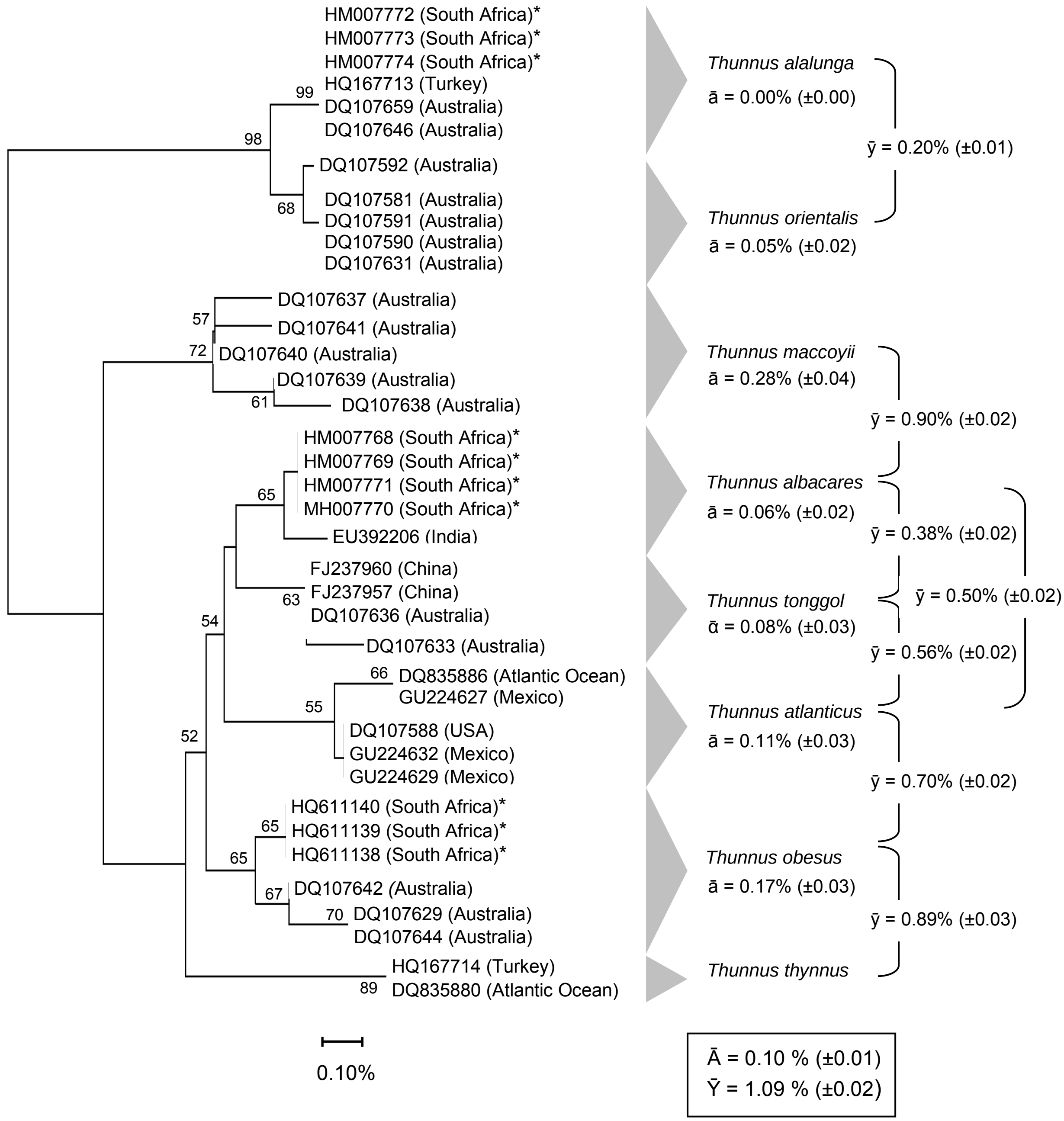


Figure 3 K2P distance neighbour-joining tree of 38 COI sequences from the eight species of tuna within the genus *Thunnus*. GenBank accession numbers and geographical origins of all specimens are provided, with asterisks indicating members of the three *Thunnus* species examined in this study. Bootstrap values higher than 50 are shown. Mean distances between members of each species (ā) are provided, as is the mean conspecific distance found overall for all eight species (Ā). The mean congeneric distance between species and their nearest neighbours are indicated (ȳ), as well as the overall mean congeneric distance found for all eight species (Ȳ).

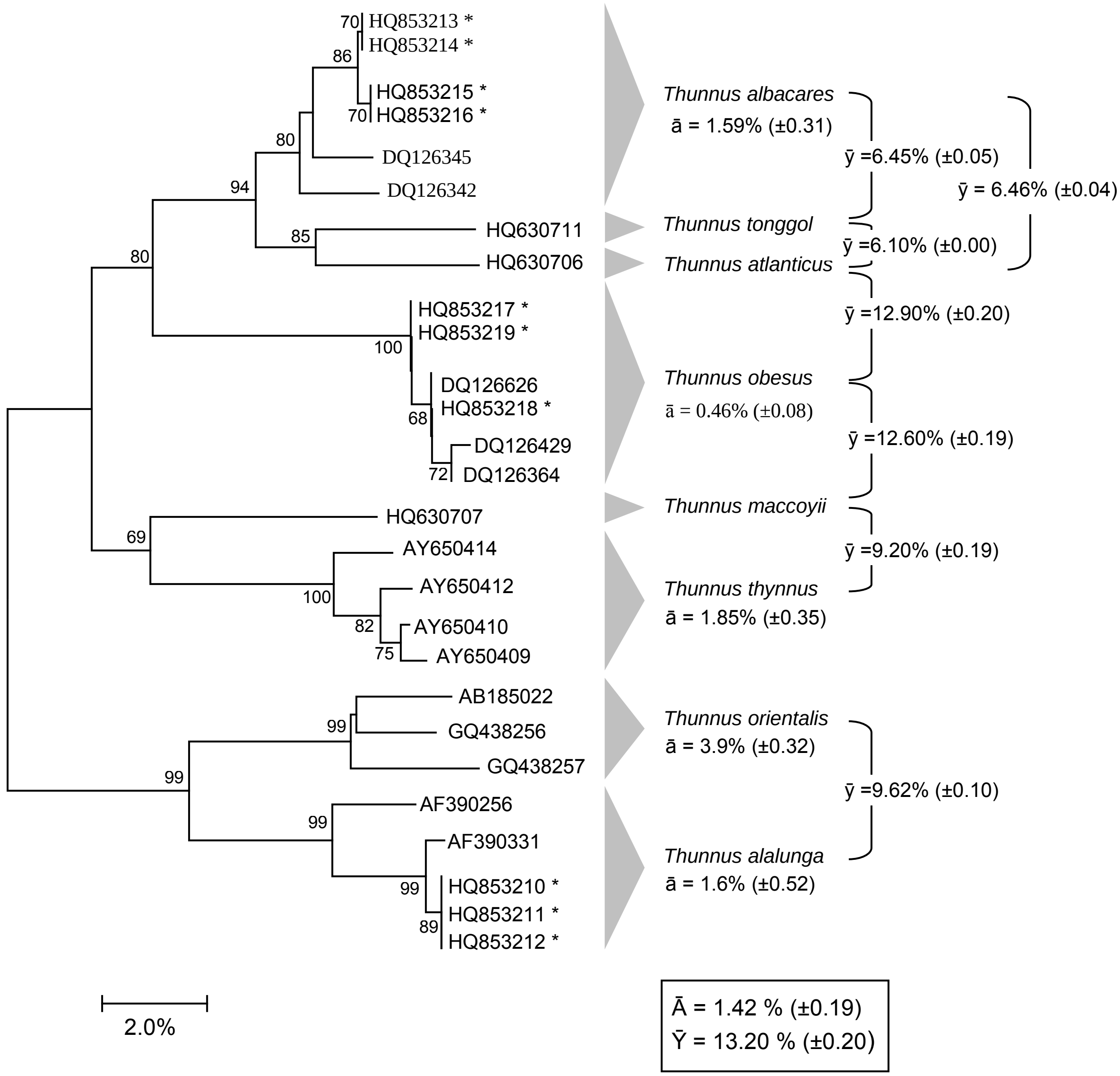


Figure 4 K2P distance neighbour-joining tree of 27 mtDNA control region sequences from the eight species of tuna within the genus *Thunnus*. GenBank accession numbers of all specimens are provided, with asterisks indicating members of the three *Thunnus* species examined in this study. Bootstrap values higher than 50 are shown. Mean distances between members of each species (ā) are provided, as is the mean conspecific distance found overall for all eight species (Ā). The mean congeneric distance between species and their nearest neighbours are indicated (ȳ), as well as the overall mean congeneric distance found for all eight species (Ȳ).

region sequences, which showed a mean distance of 9.62%. The three members of the subgenus *Neothunnus* grouped together in the control region phenogram, showing a higher level of bootstrap support (86%) than that seen in the COI tree for these species (54%). In contrast to the findings based on the COI region, the maximum conspecific distance found for all eight species (4.4%) was lower than the minimum congeneric distance (6.1%) seen between species.

The discriminatory power of the control region has been shown to be superior to the COI region for the species of *Thunnus* examined in this study. Nonetheless, introgression between several members of this genus is known to occur (Chow & Kishino, 1995; Alvarado Bremer *et al*., 1997; 2005; Viñas & Tudela, 2009) and Chow and Kishino (1995) proposed that mtDNA sequence data alone would not be sufficient for differentiating *Thunnus* species if horizontal transfer of mtDNA exists. In this context, Viñas and Tudela (2009) showed that mtDNA control region sequencing could not discriminate between *T. alalunga* and introgressed members of *T. thynnus* showing albacore-like sequences. These authors recommended that the sequencing of a nuclear fragment, the ribosomal DNA first internal transcribed spacer (ITS1), was more suitable for differentiating the aforementioned *Thunnus* specimens. Therefore, it appears that while sequencing of the mtDNA control region can serve as an extremely useful tool for *Thunnus* species identification, this will likely require supplementary sequencing of a nuclear DNA locus (such as the ITS1) if potentially introgressed members of this genus are to be revealed.

Conclusions

To our knowledge, this is the first study that has sought to establish a comprehensive DNA sequence database, including COI barcoding records, for those fish species commonly encountered on the South African market. In particular, the utility of DNA barcoding has been demonstrated and the results suggest that the COI region could serve as a single genetic marker to discriminate the vast majority of the fish species included in this study, with only members of the genus *Thunnus* likely requiring further confirmation through the use of mitochondrial control region sequencing and possibly also nuclear DNA analysis. The patterning of divergence in the mtDNA regions examined has revealed close correspondence with those species assigned through prior morphological analyses, emphasising the value of integrating DNA-based methodologies with classical identification approaches to validate existing taxonomic systems. In addition, the uncovering of a potentially cryptic species of *T. atun* in this work has shown the potential of DNA barcoding in revealing overlooked diversity and for highlighting taxa which require additional investigation and resolution.

Overall, the results and the DNA sequences generated from this research have provided the necessary genetic information to allow the explicit identification of 53 commercially important fish species in South Africa. Aside from permitting identification of whole fish specimens, these developed systems should also be extendable to the identification of fish at any developmental stage, from eggs to adulthood, and for body fragments. A realm of applications has consequently been opened relating to conservation, ecology research, control of commercial practices and detection of retail fraud, from which the entire fisheries supply chain, regulators and inevitably consumers will undoubtedly benefit. Such work should prove pertinent within an environment where both natural and human-induced activities are rapidly accelerating modifications in the abundance and distribution of fish species.

References

Allen, R., Joseph, J. & Squires, D. (2010). *Conservation and Management of Transnational Tuna Fisheries*. Pp. 3-87. New Jersey, USA: John Wiley & Sons Inc.

Atkins, H. (2010). You could be eating shark meat and not even know it. *Cape Times*, May 14, 2010.

Alvarado Bremer, J.R. (1994). Assessment of morphological and genetic variation of the swordfish (*Xiphias gladius* Linnaeus): evolutionary patterns of nucleotide substitution in the mitochondrial genome. PhD Thesis, University of Toronto, Canada (As cited by Viñas & Tudela, 2009).

Alvarado Bremer, J.R., Naseri, I. & Ely, B. (1997). Orthodox and unorthodox phylogenetic relationships among tunas revealed by the nucleotide sequence analysis of the mitochondrial control region. *Journal of Fish Biology*, **50**, 540-554.

Alvarado Bremer, J.R., Viñas, J., Mejuto, J., Ely, B. & Pla, C. (2005). Comparative phylogeography of Atlantic bluefin tuna and swordfish: the combined effects of vicariance, secondary contact, introgression, and population expansion on the regional phylogenies of two highly migratory pelagic fishes. *Molecular Phylogenetics and Evolution*, **36**, 169-187.

Blaxter, M.L. (2004). The promise of a DNA taxonomy. *Philosophical Transactions of the Royal Society B*, **359**, 669-679.

Cawthorn, D.M., Steinman, H.A. & Witthuhn, R.C. (2011a). Evaluating the availability of fish species on the South African market and the factors undermining sustainability and consumer choice. *Food Control*, **22**, 1748-1759.

Cawthorn, D.M., Steinman, H.A. & Witthuhn, R.C. (2011b). Comparative study of different methods for the extraction of DNA from fish species commercially available in South Africa. *Food Control*, **22**, 231-244.

Chow, S. & Kishino, H. (1995). Phylogenetic relationships between tuna species of the genus *Thunnus* (Scombridae: Teleostei): inconsistent implications from morphology, nuclear and mitochondrial genomes. *Journal of Molecular Evolution*, **41**, 741-748.

Collette, B.B. (1978). Adaptations and systematics of the mackerels and tunas. In: *The Physiological Ecology of Tunas* (edited by G.D. Sharp & A.E. Dizon). Pp. 7-39. London, UK: Academic Press.

Collette, B.B., Reeb, C. & Block, B.A. (2001). Systematics of the tunas and mackerels (Scombridae). In: *Tuna: Physiology, Ecology and Evolution* (edited by B.A. Block & E.D. Stevens). Pp. 5-30. London, UK: Academic Press.

Dasmahapatra, K.K. & Mallet, J. (2006). DNA barcodes: recent successes and future prospects. *Heredity*, **97**, 254-255.

Dawnay, N., Ogden, R., McEwing, R., Carvalho, G.R. & Thorpe, R.S. (2007). Validation of the barcoding gene COI for use in forensic genetic species identification. *Forensic Science International*, **173**, 1-6.

Di Finizio, A., Guerriero, G., Russo, G.L. & Ciarcia, G. (2007). Identification of gadoid species (Pisces, Gadidae) by sequencing and PCR–RFLP analysis of mitochondrial 12S and 16S rRNA gene fragments. *European Food Research and Technology*, **225**, 337-344.

Elias, M., Hill, R.I., Willmott, K.R., Dasmahapatra, K.K., Brower, A.V.Z., Mallet, J. & Jiggins, C.D. (2007). Limited performance of DNA barcoding in a diverse community of tropical butterflies. *Proceedings of the Royal Society B*, **274**, 2881-2889.

Elliot, N.G. & Ward, R.D. (1995). Genetic relationships of eight species of Pacific tunas (Teleostei: Scombridae) inferred from allozyme analysis. *Marine and Freshwater Research*, **46**, 1021-1032.

FAO (Food and Agriculture Organization of the United Nations) (2009). *FAO Yearbook of Fishery and Aquaculture Statistics 2007*. Pp. 1-72. Rome, Italy: FAO.

FAO (Food and Agriculture Organization of the United Nations) (2010). Fishery and aquaculture country profiles: South Africa national fishery sector overview. [Internet document]. URL www.fao.org/fishery/countrysector/FI-CP_ZA/en. 12 July 2010.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783-791.

Froese, R. & Pauly, D. (2010). FishBase. World Wide Web electronic publication, version 01/2010. [Internet document]. URL www.fishbase.org. 21 January 2011.

FSA (Food Standards Agency) (2000). Survey of misdescription of tuna species in tuna products. [Internet document]. URL www.food.gov.uk/science/surveillance/fsis-2000/01tuna. 10 November 2010.

Gil, L.A. (2007). PCR-based methods for fish and fishery products authentication. *Trends in Food Science and Technology*, **18**, 558-566.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & deWaard, J.R. (2003a). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B*, **270**, 313-321.

Hebert, P.D.N., Ratnasingham, S. & deWaard, J.R. (2003b). Barcoding animal life: cytochrome *c* oxidase subunit I divergences among closely related species. *Proceedings of the Royal Society B*, **270**, S96-S99.

Hebert, P.D.N., Stoeckle, M.Y., Zemlak, T.S. & Francis, C.M. (2004). Identification of birds through DNA barcodes. *PLoS Biology*, **2**, 1657-1663.

Hubalkova, Z., Kralik, P., Tremlova, B. & Rencova, E. (2007). Methods of gadoid fish species identification in food and their economic impact in the Czech Republic: a review. *Veterinarni Medicina*, **52**, 273-292.

Ivanova, N.V., Zemlak, T.S., Hanner, R. & Hebert, P.D.N. (2007). Universal primer cocktails for fish DNA barcoding. *Molecular Ecology Notes*, **7**, 544-548.

Jacquet, J.L. & Pauly, D. (2008). Trade secrets: renaming and mislabelling of seafood. *Marine Policy*, **32**, 309-318.

Kimura, M. (1980). A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**, 111-120.

Lakra, W.S., Verma, M.S., Goswani, M., Lal, K.K., Mohindra, V., Punia, P., Gopalakrishnan, A., Singh, K.V., Ward, R.D. & Hebert, P. (2011). DNA barcoding Indian marine fishes. *Molecular Ecology Resources*, **11**, 60-71.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J. & Higgins, D.G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, **23**, 2947-2948.

Lockley, A.K. & Bardsley, R.G. (2000). DNA-based methods for food authentication. *Trends in Food Science and Technology*, **11**, 67-77.

Lowenstein, J.H., Amato, G. & Kolokotronis, S.O. (2009). The real *maccoyii*: identifying tuna sushi with DNA Barcodes - contrasting characteristic attributes and genetic distances. *PLoS One*, **4**, e7866.

Martinez, I., James, D. & Loréal, H. (2005). Application of modern analytical techniques to ensure seafood safety and authenticity. In: *FAO Fisheries Technical Paper No. 455*. Pp. 1-73. Rome, Italy: FAO.

Messing, J. (1983). New M13 vectors for cloning. *Methods in Enzymology*, **101**, 20-78.

Rasmussen, R.S. & Morrisey, M.T. (2009). Application of DNA-based methods to identify fish and seafood substitution on the commercial market. *Comprehensive Reviews in Food Science and Food Safety*, **8**, 118-154.

Rock, J., Costa, F.O., Walker, D.I., North, A.W., Hutchinson, W.F. & Carvalho, G.R. (2008). DNA barcodes of fish of the Scotia Sea, Antarctica indicate priority groups for taxonomic and systematics focus. *Antarctic Science*, **20**, 253-262.

Rokas, A., Ladoukakis, E. & Zouros, E. (2003). Animal mitochondrial DNA recombination revisited. *Trends in Ecology and Evolution*, **18**, 411-417.

Safina, C. (2001). Tuna conservation. In: *Tuna: Physiology, Ecology and Evolution* (edited by B.A. Block & E.D. Steven). Pp. 414-461. London, UK: Academic Press.

Smith, J.L.B. & Smith, M.M. (1966). *Fishes of the Tsitsikamma Coastal National Park*. P 102. Pretoria, South Africa: National Parks Board Trust.

Steinke, D., Zemlak, T.S., Hebert, P.D.N. & Adamowicz, S.A. (2009a). DNA barcoding of Pacific Canada's fishes. *Marine Biology*, **156**, 2641-2647.

Steinke, D., Zemlak, T.S. & Hebert, P.D.N. (2009b). Barcoding Nemo: DNA-based identifications for the ornamental fish trade. *PLoS One*, **4**, e6300.

Stoeckle, M. (2003). Taxonomy, DNA, and the bar code of life. *BioScience*, **53**, 796-797.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, **24**, 1596-1599.

Teletchea, F. (2009). Molecular identification methods of fish species: reassessment and possible applications. *Reviews in Fish Biology and Fisheries*, **19**, 265-293.

Viñas, J. & Tudela, S. (2009). A validated methodology for genetic identification of tuna species (genus *Thunnus*). *PLoS One*, **4**, e7606.

Von der Heyden, S., Barendse, J., Seebregts, A.J. & Matthee, C.A. (2010). Misleading the masses: detection of mislabelled and substituted frozen fish products in South Africa. *ICES Journal of Marine Science*, **67**, 176-185.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R. & Hebert, P.D.N. (2005). DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B,* **360**, 1847-1857.

Ward, R.D. & Holmes, B. (2007). An analysis of nucleotide and amino acid variability in the barcode region of cytochrome *C* oxidase I (*cox1*) in fishes. *Molecular Ecology Notes*, **7**, 899-907.

Ward, R.D., Holmes, B.H., White, W.T. & Last, P.R. (2008). DNA barcoding Australasian chondrichthyans: results and potential uses in conservation. *Marine and Freshwater Research*, **59**, 57-71.

Ward, R.D., Hanner, R. & Herbert, P.D.N. (2009). The campaign to DNA barcode all fishes, FISH-BOL. *Journal of Fish Biology*, **74**, 329-356.

Wong, E.H.K. & Hanner, R.H. (2008). DNA barcoding detects market substitution in North American seafood. *Food Research International*, **41**, 828-837.

Data Accessibility

DNA sequences

GenBank accessions HM007674 - HM007801; HQ611082 - HQ611140; HQ641664 - HQ641670; HQ853210 - HQ853219.

BOLD accessions BARFS001-09; BARFS002-10 - BARFS194-10.

Phylogenetic data

TreeBASE study accession no. S11583. Study accession URL: http://purl.org/phylo/-treebase/phylows/study/TB2:S1158.

CHAPTER 7

DNA BARCODING REVEALS A HIGH INCIDENCE OF FISH SPECIES MISREPRESENTATION AND SUBSTITUTION ON THE SOUTH AFRICAN MARKET

Abstract

The mislabelling of fishery products has emerged as a serious problem on global markets, raising the need for the development of analytical tools for species authentication. DNA barcoding, based on the sequencing of a standardised region of the cytochrome *c* oxidase I (COI) gene, has received considerable attention as an accurate and broadly applicable tool for animal species identifications. The aim of this study was to investigate the utility of DNA barcoding for the identification of a variety of commercial fish in South Africa and, in so doing, to estimate the prevalence of species substitution and fraud prevailing on this market. A *ca*. 650 base pair (bp) region of the COI gene was sequenced from 248 fish samples collected from seafood wholesalers and retail outlets in South Africa, following which species identifications were made in the Barcode of Life Database (BOLD) and in GenBank. DNA barcoding was able to provide unambiguous species-level identifications for 235 of 248 (95%) samples analysed. Overall, 10 of 108 (*ca.* 9%) samples from wholesalers and 43 of 140 (*ca.* 31%) from retailers were identified as different species to the ones indicated at the point of sale. Although some cases of mislabelling were potentially unintentional due to misapplied market nomenclature, a far greater proportion represented serious and seemingly deliberate acts of fraud for the sake of increased profits. This study has highlighted that the existing legislation pertaining to seafood marketing in South Africa is inadequate or poorly enforced and requires urgent revision. In the light of the results presented here, DNA barcoding appears to hold great potential for fish authentication monitoring by both regulatory bodies and industry, the utilisation of which could enhance transparency and fair trade on the domestic fisheries market.

Cawthorn, D.M., Steinman, H.A. & Witthuhn, R.C. (2011). DNA barcoding reveals a high incidence of fish species misrepresentation and substitution on the South African market. *Food Research International* (accepted for publication).

Introduction

The world's marine fish stocks, which were considered just over a century ago to be 'inexhaustible' (Huxley, 2007), now face extreme fishing pressure as the insatiable human appetite for seafood continually outpaces supply (Delgado *et al*., 2003). Current data indicate that widespread overfishing has fully exploited, over-exploited or depleted up to 75% of global fish stocks (FAO, 2009) and has had deleterious effects on aquatic ecosystems (Pauly *et al*., 2005; Worm *et al*., 2006). In a pertinent four-year study on 10 large marine ecosystems around the world, Worm *et al*. (2009) reported that 63% of the assessed fish stocks were below desired levels and still require rebuilding, in spite of the numerous restrictions (annual harvest quotas, rights allocations, fishing gear modifications and seasonal or area closures) that have been imposed to promote more sustainable fisheries management (Beddington *et al*., 2007; Brunner *et al*., 2009).

During the last two decades, there has been a growing realisation that the incorporation of consumer behaviour into marine conservation strategies will be required if the trends in fisheries declines are to be reversed (Kaiser & Edwards-Jones, 2006). This realisation has led to a number of sustainable seafood awareness campaigns being initiated in many parts of the world, including the United Kingdom (UK), United States (US), Australia and Canada. The Southern African Sustainable Seafood Initiative (SASSI) was established in 2004 with similar aims of educating the local population on marine conservation issues and shifting consumer choices towards more sustainable seafood species. Typically, such organisations compile seafood lists that rank species according to sustainability criteria (e.g. 'best choice' or 'avoid'), the details of which are publicly disseminated via wallet cards, electronic databases and mobile phone applications (Roheim & Sutinen, 2006). A fundamental requirement for the success of all consumer awareness campaigns, as well as for fisheries management in general, is the accurate naming and labelling of fish products at the point of sale. Unfortunately, with escalating demand and globalisation of seafood trade, the current market climate in many countries is highly conducive to fraud and mislabelling of fish products (Jacquet & Pauly, 2007).

The mislabelling of fish species can manifest in several forms, as well as at any stage in the fisheries supply chain (Logan *et al*., 2008). A portion of the mislabelling that occurs is likely unintentional as fish species identities may be easily mistaken. Confusion may also arise due to the fact that different fish species can be referred to by a common vernacular name, or by different vernacular names in different regions (Buck, 2009). Of greater concern, however, is that some unscrupulous traders may deliberately use mislabelling as a means to launder illegally-caught fish into legitimate markets, or simply to defraud consumers for the purpose of accruing greater profits (Ogden, 2008). Since the flesh of many fish species is similar in appearance, taste and texture, it becomes relatively easy for species of high commercial value to be substituted, either partially or entirely, with species of lower value. The lack of traceability in the fisheries supply chain also provides a considerable opportunity for mislabelling. Fish products often change hands several times on route from the fishing vessels to the consumer's plate, making it difficult to identify the link in the supply chain where the fraud or substitution occurred (Thompson *et al*., 2005).

Whether accidental or deliberate, fish mislabelling is not only a form of economic deception, but it also undermines the efforts of seafood awareness campaigns and can further erode already threatened fisheries (Jacquet & Pauly, 2007). For instance, 77% of the fish labelled as 'red snapper' in the US have been found to be substituted with less expensive and/or overexploited species (Marko *et al*., 2004). In South Africa, shortfin mako shark has been sold as 'ocean fillets' or 'sokomoro' to increase its appeal (Atkins, 2010), even though it is listed as 'vulnerable' by the International Union for Conservation of Nature (IUCN, 2010). Furthermore, just as ichthyologic name-swapping can prevent consumers from making choices in favour of conservation, it also infringes on their right to safeguard their own health. Certain fish species can cause fatal allergic reactions (Triantafyllidis *et al*., 2010), while others contain potent toxins or high levels of contaminants. Reports have emerged on the mislabelling of pufferfish and oilfish as 'monkfish' and 'cod', respectively, where both cases have caused serious illness (Lam, 2007; Cohen *et al*., 2009).

Government regulations in many countries, including South Africa, require the full disclosure of food product content and stipulate that food labelling must not be misleading (NRCS, 2003; Martinez *et al*., 2005; DoH, 2010). Nevertheless, such provisions have done little to deter mislabelling as they are often poorly enforced, or because the penalties for non-compliance are small in comparison to the profits resulting from fraudulent fish trading (Buck, 2009). There is now mounting evidence that molecular species identification methods, particularly those based on DNA analysis, can serve as critical tools for industry self-regulation, governmental monitoring and prosecution of illegal activates (Ogden, 2008). In particular, DNA barcoding — the sequencing of an approximately 650 base pair (bp) region of the cytochrome *c* oxidase I (COI) gene — has gained widespread support in the scientific literature as a rapid, cost effective and standardised method for the identification of a diverse range of animal lineages, including fish species (Hebert *et al*., 2003a,b; Ward *et al*., 2005). This mitochondrial DNA (mtDNA) locus has been validated as a diagnostic marker for forensic identification applications (Dawnay *et al*., 2007). In addition, COI barcoding is under consideration by the United States Food and Drug Administration (FDA) for uptake into their current regulatory framework and to serve as a replacement for the technique of protein isoelectric focusing for fish species identification (Yancy *et al*., 2008; Ward *et al*., 2009; Handy *et al*., 2011). Adoption of the COI gene for DNA barcoding purposes by the Consortium for the Barcode of Life (CBOL) has led to the initiation of a number of international collaborative research efforts, including the Fish Barcode of Life Initiative (FISH-BOL), which aims to barcode all fish species of the world (Swartz *et al*., 2008; Ward *et al*., 2009; Steinke & Hanner, 2011).

DNA barcoding has been utilised to evaluate the incidence of fish species substitutions in North America (Wong & Hanner, 2008), Europe (Miller & Mariani, 2010) and Italy (Barbuto *et al*., 2010; Filonzi *et al*., 2010). However, to date, there have been no published reports on the use of this method to estimate the prevalence of such substitutions in South Africa. Considering that South Africa plays a leading role on the African continent in terms of both fish production and trade (INFOSA, 2007), such an evaluation is imperative to determine the incidence of mislabelling that could perpetuate locally or in exported commodities. The aim of this study was to investigate the utility of DNA barcoding for the identification of a large variety of fish products commercially traded at the wholesale and retail levels in South Africa, and in so doing, to assess the extent of misrepresentation and substitution occurring on this market.

Materials and methods

Sample collection

Fish samples were collected over a two-year period (2008 - 2010) in four provinces of South Africa, namely the Western Cape (WC), Eastern Cape (EC), KwaZulu-Natal (KZN) and Gauteng (GP). The former three provinces are the major coastal fishing provinces in South Africa and were included as these were expected to have access to a large variety of locally-caught fish species. Gauteng (GP) was included in order to evaluate the commercial fish trading practices in an inland province, principally because it is the most populated province in South Africa with the highest per capita income (Schlemmer, 1998). A total of 257 samples were collected, of which 108 (42%) were obtained from the wholesaler/distributor level, while 149 (58%) were obtained from retail outlets, which included both supermarkets and fish markets. Supermarkets were defined as those stores that sold fish and various other grocery items, while fish markets were defined as those stores selling primarily seafood commodities. All samples collected from wholesalers/ distributors were purchased frozen, but these included both whole and processed specimens. Fresh, frozen, whole and processed fish samples were acquired from the retail outlets. All samples were stored in a laboratory freezer (-20°C) following collection.

DNA extraction

Tissue was excised from the lateral muscle of each fish specimen with a sterile scalpel and forceps. Total genomic DNA was extracted from *ca*. 500 mg of the muscle tissue using the SureFood® PREP Allergen Kit (r-Biopharm, supplied by AEC-Amersham, Cape Town, South Africa), following the manufacturer's instructions. The concentration and purity of the extracted DNA was assessed in a spectrophotometer (Beckman Coulter DU530, Beckman Instruments, Fullerton, USA) at 260 and 280 nm. DNA extracts were stored at -20 °C prior to further analysis.

Polymerase Chain reaction (PCR)

A 652 base pair (bp) fragment from the 5' region of the COI gene was PCR amplified using the M13-tailed primer cocktail (C_FishF1t1 / C_FishR1t1) previously described for the DNA barcoding of fish species (Ivanova *et al*., 2007). The 25 µl PCR reaction mixtures contained 2.5 µl (1 X) reaction buffer ($MgCl_2$ free) (Super-Therm, supplied by Southern Cross Biotechnologies, Cape Town, South Africa), 2.5 µl (2.5 mM) $MgCl_2$ (25 mM, Super-Therm), 0.25 µl (100 nM) of each primer (10 µM stocks), 0.125 µl (0.625 U) *Taq* DNA polymerase (5U/µl, Super-Therm), 0.5 µl (0.2 mM) of mixed dNTPs (10 mM, AB gene, supplied by Southern Cross Biotechnologies) and 2 µl (*ca*. 2 µg) of DNA template. PCR amplifications were performed in a Mastercycler Personal (Eppendorf, Germany) utilising the following thermal cycling parameters: initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 52 °C for 40 s and chain elongation at 72 °C for 60 s, followed by final extension at 72 °C for 10 min. The success of amplification was assessed by electrophoresis (90 volts, 45 min) of the PCR products in 1.5% (m/v) agarose (Sigma-Aldrich, Gauteng, South Africa) gels, with subsequent visualisation under an ultraviolet light (Vilber Lourmat, Marne La Vallee, France).

In the few cases where COI barcoding did not deliver unequivocal species resolutions for certain specimens, a *ca*. 450 bp fragment of the faster-evolving mtDNA control region (d-loop) was amplified for confirmatory purposes according to Alvarado Bremer (1994), since this region contains hypervariable sequences which are reportedly useful for analysing the nucleotide variability in both populations and closely-related species (Chow *et al*., 1997; Faber, 1997; Donaldson & Wilson, 1999; Jie *et al*., 2011). The reaction mixtures for the control region PCR (25 µl final volume) comprised 2.5 µl (1 X) reaction buffer ($MgCl_2$ free) (Super-Therm), 2.0 µl (2.0 mM) $MgCl_2$ (25 mM, Super-Therm), 1.0 µl (400 nM) of each primer (10 µM stocks), 0.10 µl (0.50 U) *Taq* DNA polymerase (5U/µl, Super-Therm), 2.0 µl (0.8 mM) of mixed dNTPs (10 mM, AB gene) and 1 µl (*ca*. 1 µg of DNA template). PCR cycling conditions included an initial denaturation step at 94 °C for 5 min, 35 cycles of 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 60 s, followed by a final extension step of 72 °C for 10 min.

Sequencing and sequence analysis

PCR amplification products were purified with the NucleoFast 96 PCR Clean-up Kit (Macherey-Nagel, supplied by Separations, Gauteng, South Africa) following the instructions of the manufacturer. Sequencing of the purified PCR products was performed using BigDye chemistry and analysis on an ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, USA). The primers for M13-tailed PCR products described by Messing (1983) were used for the sequencing of the COI amplicons, while the PCR amplification primers were used as sequencing primers for the control region amplicons. Sequences were aligned and manually edited using BioEdit sequence alignment editor, version 7.0.9.0 (Hall, 1999). The generated COI and control region sequences were identified in GenBank (www.ncbi.nlm.nih.gov) using the BLASTn search tool. Identification results for the COI sequences were cross-referenced within BOLD (www.barcodinglife.org) (Ratnasingham & Hebert, 2007). As a general rule, a top match with a sequence similarity of at least 98% was used as a criterion to designate potential species identifications (Barbuto *et al*., 2010). Species identifications made through GenBank and BOLD were compared to the market names and species names (when available) under which the queried samples had been sold. Since the COI and control region sequences generated in this study were not derived from voucher samples or expertly-identified fish specimens, these sequences were not submitted to either GenBank or BOLD.

Evaluation of species authenticity and mislabelling

For the evaluation of the accuracy of fish species labelling, a multiple-step protocol was employed to ensure that all samples were consistently evaluated in a rigid and literal manner. Since both the GenBank and BOLD databases rely on FishBase (http://www.fishbase.org) as a taxonomic authority for valid fish species names (Froese & Pauly, 2011; Wong & Hanner, 2008), top species matches (highest percentages of similarity) for each specimen were compared with the species and corresponding common names within the FishBase database. In cases where inconsistencies were found between the market names of the queried samples and the currently accepted fish names in FishBase, species and common names were cross-checked in Van der Elst (1997), Smith *et al.* (2003) and the SASSI database (www.wwfsassi.co.za). The three latter sources were used in the absence of authoritative lists in South Africa containing acceptable market names for seafood species sold on the local market (such as those available in the US Food and Drug Administration (FDA) list, www.fda.gov).

Results and discussion

The DNA extracted from 248 of the 257 (96%) collected market samples was successfully amplified with the COI primer cocktail. From these, the resulting PCR products were sequenced to obtain full length DNA barcodes averaging 650 bp in length. No insertions, deletions or stop codons were observed in any of the COI sequences, consistent with all amplified sequences being functional mitochondrial COI sequences. Additionally, the fact that all COI sequences exceeded 600 bp in length suggests that nuclear DNA sequences originating from mtDNA (NUMTs) were not sequenced (vertebrate NUMTs are generally smaller than 600 bp) (Zhang & Hewitt, 1996).

Nine samples, constituting canned products labelled as ‘tuna chunks’, ‘pink salmon’ and ‘mackerel’, did not amplify with the COI cocktail and were therefore not included in further analyses. This amplification failure was not anticipated to have been due to incompatible PCR primers, as barcode sequences for all of the nominal species comprising these samples have been successfully amplified in the past and are available in BOLD and GenBank (Ward *et al*., 2005; Rasmussen *et al*., 2009). Rather, it is well established that extensive processing conditions, such as the thermal treatments used in canning operations, can lead to the degradation of DNA into fragments smaller than 200 bp (Ram *et al*., 1996; Quinteiro *et al*., 1998; Pardo & Pérez-Villareal, 2004). Consequently, poor DNA quality in these nine samples is the most likely explanation for the failure in PCR amplification. Universal primers targeting shorter fragments of the COI gene (described as 'mini barcodes') have been reported for the identification of specimens whose DNA is expected to be degraded (Hajibabaei *et al*., 2006; Meusnier *et al*., 2008). Such methods may be more suitable for the authentication of highly processed fish samples, such as those that failed to amplify in this study using the conventional COI primer cocktail for fish DNA barcoding.

For all 248 generated COI sequences, maximum species identities in the range of 98 - 100% were obtained in GenBank and/or BOLD. For two specimens identified in BOLD as *Coryphaena equiselis* and *Etelis coruscans* (Table 1), corresponding COI sequences were not available in GenBank to permit species identifications. Additionally, in the large majority of cases where sequence similarities below 100% were attained, BOLD yielded superior species resolutions in comparison to those achieved in GenBank. Although barcode data from BOLD and GenBank are continuously exchanged, a possible reason for this finding is that the BOLD database currently contains a wider representation of COI sequences with which unknown specimens can be compared (Wong & Hanner, 2008). As of March 2011, over one million barcode sequences were contained within BOLD, while a search for available COI sequences in GenBank returned about half this number. DNA barcoding permitted explicit species resolution for 235 of the 248 (95%) sequenced samples. Only *Helicolenus dactylopterus* and two species of the genus *Thunnus* showed overlapping COI barcodes with congeneric species and could not be unequivocally differentiated on this basis. The non-coding mtDNA control region was recognized as the most promising marker for the confirmatory identification of these closely-related fish species since it evolves more rapidly than the coding regions of the mtDNA (such as the COI and cytochrome *b* genes) and it is reported to be the most variable segment in the mitochondrial genome (Brown *et al*., 1993; Zhao *et al*., 2006; Chauhan & Rajiv, 2010; Jie *et al*., 2011). In addition, since control region sequences have been frequently employed for intra- and interspecific genetic studies of fishes (Lee *et al*., 1995; Alvarado Bremer *et al*., 1997; Carlsson *et al*., 2004; Aboim *et al*., 2005; Smith *et al*., 2009; Viñas & Tudela, 2009; Cawthorn *et al*., 2011), it was anticipated prior to analyses that reference sequences for this region would be readily available in DNA sequence repositories to allow identification of species not discriminated by COI sequencing.

Overall, 53 of the 248 (*ca*. 21%) fish samples analysed from all outlets were genetically identified as different species to the ones indicated and could consequently be considered mislabelled (Tables 1 and 2). The frequency of fish species mislabelling observed here is comparable to those results obtained from similar studies in Europe (Miller & Mariani, 2010), Italy (Barbuto *et al*., 2010; Filonzi *et al*., 2010) and North America (Marko *et al*., 2004; Logan *et al*., 2008; Wong & Hanner, 2008), emphasising that this problem is pervasive on a worldwide scale. A surprising finding emerging from this study was the large discrepancy in the incidence of mislabelling manifesting at the wholesale and retail levels in South Africa. Mislabelling was found to be considerably more pronounced in retail outlets than in wholesale outlets and both sets of results are subsequently discussed in turn.

Mislabelling at the wholesale level

Discrepancies were found between the identified species and the declared market names for 10 of the 108 (*ca*. 9%) frozen fish samples obtained from seafood wholesalers/ distributors in South Africa (Table 1). Although the currently available literature suggests that most species mislabelling transpires following procurement from the fishermen for the sake of increased profits (Jacquet & Pauly, 2008), it is, nevertheless, quite conceivable that some of this mislabelling may have occurred prior to fish receipt by the respective wholesalers or distributors. As suggested by Logan *et al*. (2008), a factor potentially contributing to this problem is the loss of clear identification characteristics of fish specimens between the time of capture and the time that these appear for sale. The results obtained in this study indeed indicated that the majority of species substitutions at the wholesale level involved fish products that had been processed to some degree, meaning that the distinguishing morphological characteristics had been damaged or removed. Nine of the 94 (*ca*. 10%) filleted or processed samples were mislabelled, compared to just one of the 14 (*ca*. 7%) whole samples.

Table 1 Identification results based on cytochrome *c* oxidase I (COI) and control region (CR) sequencing for 108 fish samples from fish wholesalers and distributors in South Africa, where N indicates the number of fish samples that were correctly labelled or potentially mislabelled. Cases of suspected mislabelling are indicated in bold typescript, while GenBank Accession numbers in italics indicate sequences derived from fish specimens caught in South African waters.

Fish marketed as: (expected species)	N	Process state	Gene target	BOLD		GENBANK		
				Species identification	Similarity (%)	Species identification	Similarity (%)	Accession Number
Angelfish (*Brama brama*)	3	Filleted	COI	*Brama brama*	100	*Brama brama*	100 100	EF609300 *HQ611085*
Alaskan salmon (*Oncorhynchus keta*)	2	Filleted	COI	*Oncorhynchus keta*	100	*Oncorhynchus keta*	100	EU525057
Atlantic salmon (*Salmo salar*)	5	Filleted	COI	*Salmo salar*	100	*Salmo salar*	100	AF133701
Barramundi (*Lates calcarifer*)	**5**	**Filleted**	**COI**	***Seriolella brama*** **(common warehou)**	**100**	***Seriolella brama*** **(common warehou)**	**100**	**AY899437**
Black butterfish (*Schedophilus velaini*) [b]	2	Filleted	COI	*Hyperoglyphe moselii* [a] *Schedophilus velaini*	100 100	*Hyperoglyphe moselii* [a] *Schedophilus velaini*	100 100	DQ107610 *HQ611131*
Black ruff (*Centrolophus niger*)	2	Filleted	COI	*Centrolophus niger*	100	*Centrolophus niger*	100	AB205434
Bluenose (*Hyperoglyphe antarctica*)	1	Filleted	COI	*Hyperoglyphe antarctica*	100	*Hyperoglyphe antarctica*	100	DQ107615
	3	**Filleted**	**COI**	***Hyperoglyphe moselii*** [a] ***Schedophilus velaini*** **(violet warehou)**	**100** **100**	***Hyperoglyphe moselii*** [a] ***Schedophilus velaini*** **(violet warehou)**	**100** **100**	**DQ107610** ***HQ611131***
Blue warehou (*Seriolella brama*)	1	Filleted	COI	*Seriolella brama*	100	*Seriolella brama*	100	AB205439
Butterfish (*Ruvettus pretiosus* / *Lepidocybium flavobrunneum*)	4	Filleted	COI	*Ruvettus pretiosus*	100	*Ruvettus pretiosus*	100	EU752173
	1	Filleted	COI	*Lepidocybium flavobrunneum*	100	*Lepidocybium flavobrunneum*	100	FJ605745
Cape dory (*Zeus capensis*)	2	Whole	COI	*Zeus capensis*	100	*Zeus capensis*	100	*HM007762*
Cape gurnard (*Chelidonichthys capensis*)	2	Whole	COI	*Chelidonichthys capensis*	100	*Chelidonichthys capensis*	100	*HM007756*
Cape salmon (*Atractoscion aequidens*)	3	Filleted	COI	*Atractoscion aequidens*	100	*Atractoscion aequidens*	100	*GU946593*
Cardinal (*Epigonus telescopus*)	3	Filleted	COI	*Epigonus telescopus*	100	*Epigonus telescopus*	100 99	*HM007701* EF609350
Deep-water Cape hake (*Merluccius paradoxus*)	3	Whole	COI	*Merluccius paradoxus*	100	*Merluccius paradoxus*	100 100	GU324176 *HM007683*
Dorado (*Coryphaena hippurus*)	5	Filleted	COI	*Coryphaena hippurus*	100	*Coryphaena hippurus*	100 100	DQ885089 *HM007705*
	1	**Whole**	**COI**	***Coryphaena equiselis*** **(Pompano dolphinfish)**	**99.7**	**No COI sequences for *C. equiselis* are available in GenBank**		
East coast sole (*Austroglossus pectoralis*)	3	Dressed	COI	*Austroglossus pectoralis*	100	*Austroglossus pectoralis*	99 99	EU513717 *HM007679*
Flame snapper (*Etelis coruscans*)	1	Filleted	COI	*Etelis coruscans*	99.7	No COI sequences for *E. coruscans* are available in GenBank		
Gastora (*Gasterochisma melampus*)	3	Filleted	COI	*Gasterochisma melampus*	100	*Gasterochisma melampus*	100 100	DQ107691 *HM007708*

Table 1 (continued)

Fish marketed as: (expected species)	N	Process state	Gene target	BOLD Species identification	BOLD Similarity (%)	GENBANK Species identification	GENBANK Similarity (%)	GENBANK Accession Number
Japanese amberjack (*Seriola quinqueradiata*)	1	Filleted	COI	*Seriola quinqueradiata*	100	*Seriola quinqueradiata*	100	HQ641665
Kippers (*Clupea harengus*)	3	Filleted	COI	*Clupea harengus*	100	*Clupea harengus*	99-100	GU324181
Kingklip (*Genypterus capensis*)	12	Filleted	COI	*Genypterus capensis*	100	*Genypterus capensis*	99-100	*HM007746*
Marlin (*Makaira* spp.)	1	Filleted	COI	*Makaira nigricans* *Makaira mazara*	100 100	*Makaira nigricans* *Makaira mazara*	99 99	GQ202124 AB470304
	1	**Filleted**	**COI**	***Tetrapturus angustirostris* (shortbill spearfish)**	**99.7**	***Tetrapturus angustirostris* (shortbill spearfish)**	**99**	**AB470303**
Monk (*Lophius vomerinus*)	2	Filleted	COI	*Lophius vomerinus*	100	*Lophius vomerinus*	99 100	EU683994 *HM007765*
New Zealand ling (*Genypterus blacodes*)	2	Filleted	COI	*Genypterus blacodes*	100	*Genypterus blacodes*	99 100	HQ611135 EU074430
Pilchard (*Sardinops sagax*)	2	Whole	COI	*Sardinops sagax*	100	*Sardinops sagax*	99 100	FJ165127 *HQ611132*
Shallow-water Cape hake (*Merluccius capensis*)	4	Whole	COI	*Merluccius capensis*	100	*Merluccius capensis*	100 100	GQ988405 *HM007690*
Silver kob / Kabeljou (*Argyrosomus inodorus*)	2	Filleted	COI	*Argyrosomus inodorus*	100	*Argyrosomus inodorus*	100	*HM007711*
Snoek / barracouta (*Thyrsites atun*)	3	Filleted	COI	*Thyrsites atun*	100	*Thyrsites atun*	100	*HQ611106*
	3	Filleted	COI	*Thyrsites atun*	100	*Thyrsites atun*	99 100	EU263813 *HQ641670*
Spanish mackerel (*Scomberomorus commerson*)	2	Filleted	COI	*Scomberomorus commerson*	100	*Scomberomorus commerson*	100 100	DQ885055 *HM007790*
West coast sole (*Austroglossus microlepsis*)	8	Dressed	COI	*Austroglossus microlepis*	100	*Austroglossus microlepis*	100	*GU946575*
Yellowtail (*Seriola lalandi*)	3	Filleted	COI	*Seriola lalandi*	100	*Seriola lalandi*	100 99	*HM007727* EF609460
Yellowfin tuna (*Thunnus albacares*)	3	Filleted	COI	*Thunnus albacares* *Thunnus atlanticus* *Thunnus obesus* *Thunnus tonggol*	100 99.85 99.85 99.69	*Thunnus albacares* *Thunnus atlanticus* *Thunnus obesus* *Thunnus tonggol*	100 99 99 99	EF609629 DQ107588 DQ107642 DQ107634
			CR	------------------	------	*Thunnus albacares*	99	AF301200
	1	Filleted	COI	*Thunnus obesus* *Thunnus atlanticus* *Thunnus albacares* *Thunnus thynnus* *Thunnus tonggol*	100 100 99.8 99.5 99.5	*Thunnus obesus* *Thunnus albacares* *Thunnus thynnus* *Thunnus tonggol*	100 99 99 99	GU451754 GU256528 GU451772 DQ107634
			CR	------------------	------	*Thunnus obesus*	99	DQ126626

[a] *Hyperoglyphe moselii* and *Schedophilus velaini* refer to the same species, although *S. velaini* is the currently accepted name.

Some of the substitutions detected at the wholesaler/distributor level appeared to be more blatant than others. One case of potentially unintentional substitution involved a sample labelled as 'dorado' (*Coryphaena hippurus*), which exhibited 100% sequence similarity in BOLD with Pompano dolphinfish (*Coryphaena equiselis*) (Table 1). Both of these congeneric species occur worldwide in tropical or sub-tropical waters and it has been reported that *C. equiselis* is frequently misidentified as juvenile or female *C. hippurus* (Froese & Pauly, 2011). Somewhat less likely to be unintentional was the misrepresentation of confamilial species, such as the substitution of marlin with shortbill spearfish (*Tetrapturus angustirostris*). In addition, three samples labelled as 'bluenose' showed a 100% sequence similarity with another member of the Centrolophidae family, namely *Schedophilus velainii* (violet warehou/black butterfish) (Table 1). Although marlin and shortbill spearfish may be targeted by the same fisheries, as may also be the case for bluenose and violet warehou, the morphological characteristics of these species should allow for relatively simple discrimination when the specimens are in their whole forms.

A major case of apparently deliberate fraud detected at the wholesaler/distributor level concerned the substitution of five samples marketed as the highly-valued 'Australian barramundi', expected to be *Lates calcarifer*, but identified by COI sequencing as the lower-valued common warehou (*Seriolella brama*) (Table 1). Although all five samples tested were derived from the same distributor, these were representative of different batches, suggesting that the species substitution was not an isolated incident. A further four samples of the same product derived from retail outlets in all four provinces surveyed also showed 100% sequence similarity with *S. brama* in BOLD and GenBank (Table 2).

Mislabelling at the retail level

Of the 140 retail samples that were sequenced, 43 (*ca*. 31%) of these were found to be misnamed or mislabelled (Table 2). As seen at the wholesale level, mislabelling at the retail level appeared to be particularly problematic with processed fish products. Thirty-three of 96 (*ca*. 34%) filleted or processed samples were deemed to be mislabelled, compared to 10 of 44 (*ca*. 23%) samples sold in their whole state. The overall frequency of fish mislabelling, as well as the frequency of mislabelling per province surveyed, is presented in Figure 1. The highest incidence of fish mislabelling at the retail level was found in the coastal province of KZN, where 19 of the 34 (*ca*. 56%) samples analysed by DNA sequencing were misrepresented. Fish species substitution was the second most frequent in GP, where 8 of the 28 (29%) collected samples appeared to be mislabelled. A lack of vendor familiarity with marine fish nomenclature in an inland province may have been a factor leading to this high incidence of mislabelling in GP. However, it should also be noted that a corresponding lack of knowledge at the consumer level in an inland province could make it reasonably simple for dishonest retailers to mislabel their fish products for the sake of financial gain. The prevalence of retail-level mislabelling in the WC and EC was lower than that observed in KZN and GP, with 13 of 51 (25%) and 4 of 27 (15%) of the collected samples in the respective provinces being identified as other species (Fig. 1).

Fourteen of the 43 (*ca*. 33%) cases of mislabelling discovered were seemingly subtle in nature, representing substitutions with congeneric species. One such instance of inconspicuous mislabelling involved a sample marketed as ‘deep water hake’, for which a ‘product of South Africa’ declaration was also included on the packaging (Table 2). According to the recently published labelling regulations in South Africa (DoH, 2010), a ‘product of South Africa’ label would imply that the main ingredient of the foodstuff was derived from South Africa. ‘Deep water hake’ caught in South African waters is expected to be *Merluccius paradoxus*, however, COI sequencing revealed a 100% sequence similarity between the enclosed fish and the imported species *Merluccius productus* (North Pacific hake). Thus, this case not only involved misrepresentation at the species level, but also at the country-of-origin level. Similarly, the common name ‘yellowtail’ is expected to refer to *Seriola lalandi* (yellowtail amberjack), a fish regularly consumed on the South African market. Nonetheless, 5 of 9 retail samples labelled as ‘yellowtail’ showed a 100% sequence similarity with *Seriola quinqueradiata* (Japanese amberjack) (Table 2), a species most likely imported from Asian countries. This finding appears to indicate that retailers in South Africa may label this closely-related species as ‘yellowtail’, rather than take the risk of consumers rejecting the foreign counterpart due to unfamiliarity with its common name. Obviously, such practices are facilitated by the fact that there is no legal definition of which species constitutes a ‘yellowtail’ in South Africa. In addition, samples labelled as ‘Roman seabream’ and ‘red stumpnose’, expected to be the species *Chrysoblephus laticeps* and *Chrysoblephus gibbiceps*, respectively, were both found to be slinger seabream (*Chrysoblephus puniceus*) (Table 2). These cases of species substitution may hold economic implications, as the former members of the Sparidae family (particularly red stumpnose) normally command a higher price than the latter.

Table 2 Identification results based on cytochrome *c* oxidase I (COI) and control region (CR) sequencing for 148 fish samples from retail outlets (supermarkets and fish markets), where N indicates the number of fish samples that were correctly labelled or potentially mislabelled. Cases of suspected mislabelling are indicated in bold typescript, while GenBank Accession numbers in italics indicate sequences derived from fish specimens caught in South African waters.

Fish marketed as: (expected species)	N	Process state	Gene target	BOLD		GENBANK		
				Species identification	Similarity (%)	Species identification	Similarity (%)	Accession number
Angelfish (*Brama brama*)	2	Filleted	COI	*Brama brama*	100	*Brama brama*	100 100	EF609300 *HQ611085*
	1	**Filleted**	**COI**	***Taractichthys longipinnis*** **(big-scale pomfret)**	**99.5**	***Tarctichthys longipinnis*** **(big-scale pomfret)**	**99**	**EF609476**
Alaskan salmon (*Oncorhynchus keta*)	1	Filleted	COI	*Oncorhynchus keta*	100	*Oncorhynchus keta*	99	EU525057
Barracuda (*Sphyraena* spp.)	**1**	**Filleted**	**COI**	***Acanthocybium solandri*** **(wahoo)**	**100**	***Acanthocybium solandri*** **(wahoo)**	**99**	**DQ107693**
	1	**Filleted**	**COI**	***Thyrsites atun*** **(snoek)**	**100**	***Thyrsites atun*** **(snoek)**	**98**	**EU263814**
Barramundi (*Lates calcarifer*)	**4**	**Filleted**	**COI**	***Seriolella brama*** **(common warehou)**	**100**	***Seriolella brama*** **(common warehou)**	**100** **100**	**EF609461** **HM007731**
Black ruff (*Centrolophus niger*)	2	Filleted	COI	*Centrolophus niger*	100	*Centrolophus niger*	100	AB205434
Cape dory (*Zeus* spp.)	2	Whole	COI	*Zeus capensis*	100	*Zeus capensis*	100	*HM007762*
Cape gurnard (*Chelidonichthys capensis*)	1	Whole	COI	*Chelidonichthys capensis*	100	*Chelidonichthys capensis*	100	*HM007756*
Cape monk (*Lophius vomerinus*)	2	Filleted	COI	*Lophius vomerinus*	100	*Lophius vomerinus*	99 100	EU683994 *HM007765*
Cape salmon (*Atractoscion aequidens*)	5	Filleted	COI	*Atractoscion aequidens*	100	*Atractoscion aequidens*	100	*GU946593*
	1	**Filleted**	**COI**	***Tetrapturus angustirostris*** **(shortbill spearfish)**	**100**	***Tetrapturus angustirostris*** **(shortbill spearfish)**	**100** **100**	**DQ107621** ***HQ611111***
Cardinal (*Epigonus telescopus*)	1	Filleted	COI	*Epigonus telescopus*	99	*Epigonus telescopus*	98 99	EF609350 *HM007701*
Cod fish (*Gadus morhua*)	**1**	**Whole**	**COI**	***Brama brama*** **(Atlantic pomfret / angelfish)**	**99.7**	***Brama brama*** **(Atlantic pomfret / angelfish)**	**99** **99**	**EF609300** ***HQ611085***
Couta (*Scomberomorus commerson*)	1	Filleted	COI	*Scomberomorus commerson*	100	*Scomberomorus commerson*	100	DQ885055 *HM007790*
Deep water hake (product of South Africa) (*Merluccius paradoxus*)	1	Filleted	COI	*Merluccius paradoxus*	100	*Merluccius paradoxus*	100 100	GU324176 *HM007683*
	1	**Filleted**	**COI**	***Merluccius productus*** **(North Pacific hake)**	**100**	***Merluccius productus*** **(North Pacific hake)**	**100**	**FJ164854**
Dorado (*Coryphaena hippurus*)	3	Filleted	COI	*Coryphaena hippurus*	99-100	*Coryphaena hippurus*	99-100	GU225592 *HM007704*
East coast sole (*Austroglossus pectoralis*)	4	Dressed	COI	*Austroglossus pectoralis*	100	*Austroglossus pectoralis*	100 99	*HM007679* EU513717
Englishman (*Chrysoblephus anglicus*)	1	Whole	COI	*Chrysoblephus anglicus*	100	*Chrysoblephus anglicus*	100	*HQ611099*
Hake (*Merluccius* spp.)	3	Filleted	COI	*Merluccius paradoxus*	100	*Merluccius paradoxus*	100	*HM007683*
	2	Filleted	COI	*Merluccius capenis*	100	*Merluccius capenis*	100	*HM007690*

Table 2 (continued)

Fish marketed as: (expected species)	N	Process state	Gene target	BOLD Species identification	BOLD Similarity (%)	GENBANK Species identification	GENBANK Similarity (%)	GENBANK Accession number
Half moon rockcod (*Epinephelus rivulatus*)	**1**	**Whole**	**COI**	***Helicolenus dactylopterus*** ***Helicolenus barathri*** ***Helicolenus percoides*** ***Helicolenus hilgendorfi***	**100** **99.5** **99.5** **99.3**	***Helicolenus dactylopterus*** ***Helicolenus hilgendorfi*** ***Helicolenus barathri***	**100** **99** **99**	***HM007747*** **AP002948** **EF609370**
			CR	**---------------**	**------**	***Helicolenus dactylopterus* (blackbelly rosefish / jacopever)**	**99**	**AY563096**
Hottentot seabream (*Pachymetopon blochii*)	3	Whole	COI	*Pachymetopon blochii*	100	*Pachymetopon blochii*	100	*HM007693*
Jacopever / Jacs (*Helicolenus dactylopterus*)	3	Whole	COI	*Helicolenus dactylopterus* *Helicolenus barathri* *Helicolenus percoides* *Helicolenus hilgendorfi*	100 99.5 99.5 99.3	*Helicolenus dactylopterus* *Helicolenus hilgendorfi* *Helicolenus barathri*	100 99 99	*HM007747* AP002948 EF609370
			CR	---------------	------	*Helicolenus dactylopterus*	99	AY563096
Kabeljou (*Argyrosomus* spp.)	4	Filleted	COI	*Argyrosomus inodorus*	100	*Argyrosomus inodorus*	100	*HM007711*
Kahawai (*Arripis trutta*)	1	Filleted	COI	*Arripis trutta*	99.9	*Arripis trutta*	99	AB205452
King fish (*Carangoides* / *Caranx* spp.)	**1**	**Filleted**	**COI**	***Genypterus capensis* (kingklip)**	**99.8**	***Genypterus capensis* (kingklip)**	**99**	***HM007746***
Kingklip (*Genypterus capensis*)	9	Filleted	COI	*Genypterus capensis*	99-100	*Genypterus capensis*	99-100	*HM007746*
	3	**Filleted**	**COI**	***Genypterus blacodes* (pink cusk eel / ling)**	**100**	***Genypterus blacodes* (pink cusk eel / ling)**	**99** **100**	**EU074430** ***HQ611135***
Line fish (?)	**1**	**Filleted**	**COI**	***Atractoscion aequidens* (geelbek / Cape salmon)**	**99.8**	***Atractoscion aequidens* (geelbek / Cape salmon)**	**99**	***GU946593***
Longfin tuna (*Thunnus alalunga*)	2	Filleted	COI	*Thunnus alalunga* *Thunnus orientalis*	100 99.8	*Thunnus alalunga* *Thunnus orientalis*	100 100	DQ107659 DQ107631
			CR	---------------	------	*Thunnus alalunga*	99	AF390331
Maasbanker (*Trachurus capensis*)	2	Whole	COI	*Trachurus capensis*	100	*Trachurus capensis*	100	*HM007775*
Mackerel (*Scomber japonicus*)	2	Whole	COI	*Scomber japonicus*	100	*Scomber japonicus*	100 98	*HQ611117* EF433290
Mackerel (*Scomber scombrus*)	2	Canned	COI	Failed to amplify with COI cocktail				
Musselcracker (*Sparodon durbanensis* / *Cymatoceps nasutus*)	**2**	**Filleted**	**COI**	***Pseudopentaceros richardsoni* (pelagic armourhead)**	**100**	***Pseudopentaceros richardsoni* (pelagic armourhead)**	**99**	**DQ107734**
	1	**Filleted**	**COI**	***Hyperoglyphe antarctica* (bluenose warehou)**	**100**	***Hyperoglyphe antarctica* (bluenose warehou)**	**100**	**DQ107615**
Norwegian salmon (*Salmo salar*)	3	Filleted	COI	*Salmo salar*	100	*Salmo salar*	100	AF133701
	1	**Filleted**	**COI**	***Oncorhynchus mykiss* (rainbow trout)**	**100**	***Oncorhynchus mykiss* (rainbow trout)**	100	DQ288270

Table 2 (continued)

Fish marketed as: (expected species)	N	Process state	Gene target	BOLD		GENBANK		
				Species identification	Similarity (%)	Species identification	Similarity (%)	Accession Number
Panga (*Pterogymnus laniarius*)	2	Whole	COI	*Pterogymnus laniarius*	100	*Pterogymnus laniarius*	100	*HQ592212*
Pignose grunter (*Lithognathus lithognathus*)	**1**	**Filleted**	**COI**	***Seriola lalandi* (yellowtail amberjack)**	**99.6**	***Seriola lalandi* (yellowtail amberjack)**	**99 100**	**EF609460 *HM007727***
Pilchard (*Sardinops sagax*)	2	Whole	COI	*Sardinops sagax*	100	*Sardinops sagax*	100 100	FJ165127 *HQ611132*
Pink salmon (*Oncorhynchus gorbuscha*)	2	Canned	COI	Failed to amplify with COI cocktail				
'Red fish' (?)	**1**	**Whole**	**COI**	***Pterogymnus laniarius* (panga seabream)**	**100**	***Pterogymnus laniarius* (panga seabream)**	**100**	***HQ592212***
	1	**Whole**	**COI**	***Chrysoblephus puniceus* (slinger seabream)**	**100**	***Chrysoblephus puniceus* (slinger seabream)**	**100**	***HQ611088***
Red snapper (*Lutjanus* spp.)	**3**	**Whole**	**COI**	***Lutjanus Argentimaculatus* (river snapper)**	**99.9**	***Lutjanus Argentimaculatus* (river snapper)**	**99**	**DQ885026**
	1	**Whole**	**COI**	***Pterogymnus laniarius* (panga seabream)**	**100**	***Pterogymnus laniarius* (panga seabream)**	**100**	***HM007781***
	1	**Filleted**	**COI**	***Chrysoblephus laticeps* (Roman seabream)**	**100**	***Chrysoblephus laticeps* (Roman seabream)**	**100**	***HM007750***
Red stumpnose (*Chrysoblephus gibbiceps*)	**1**	**Filleted**	**COI**	***Chrysoblephus puniceus* (slinger seabream)**	**100**	***Chrysoblephus puniceus* (slinger seabream)**	**100**	***HQ611087***
Ribbon snoek (*Lepidopus caudatus*)	2	Filleted	COI	*Lepidopus caudatus*	100	*Lepidopus caudatus*	99 99	EU869824 *HM007784*
Roman / red roman (*Chrysoblephus laticeps*)	2	Whole	COI	*Chrysoblephus laticeps*	100	*Chrysoblephus laticeps*	100	*HM007750*
	1	**Whole**	**COI**	***Cheimerius nufar* (santer seabream)**	**100**	***Cheimerius nufar* (santer seabream)**	**100**	***HQ611102***
	1	**Whole**	**COI**	***Chrysoblephus puniceus* (slinger seabream)**	**100**	***Chrysoblephus puniceus* (slinger seabream)**	**100**	***HQ611087***
Sailfish (*Istiophorus* spp.)	**1**	**Filleted**	**COI**	***Tetrapturus angustirostris* (shortbill spearfish)**	**99.9**	***Tetrapturus angustirostris* (shortbill spearfish)**	**99 99**	**HM071007 *HQ611111***
Santer / soldier (*Cheimerius nufar*)	4	Whole	COI	*Cheimerius nufar*	99-100	*Cheimerius nufar*	99-100	*HQ611102*
Silver (*Argyrozona argyrozona*)	3	Whole	COI	*Argyrozona argyrozona*	100	*Argyrozona argyrozona*	100	*GU946638*
Skipjack tuna (*Katsuwonus pelamis*)	2	Filleted	COI	*Katsuwonus pelamis*	100	*Katsuwonus pelamis*	100 100	EU014261 *HQ611090*
Slinger (*Chrysoblephus puniceus*)	2	Whole	COI	*Chrysoblephus puniceus*	100	*Chrysoblephus puniceus*	100	*HQ611087*

Table 2 (continued)

Fish marketed as: (expected species)	N	Process state	Gene target	BOLD		GENBANK		
				Species identification	Similarity (%)	Species identification	Similarity (%)	Accession number
Snoek (*Thyrsites atun*)	2	Filleted	COI	*Thyrsites atun*	100	*Thyrsites atun*	100 100	EU263813 *HQ641670*
	1	**Filleted**	**COI**	***Scomberomorus plurilineatus*** **(Kanadi kingfish)**	**100**	**---------------**	**------**	**---------------**
Swordfish (*Xiphias gladius*)	1	Filleted	COI	*Xiphias gladius*	100	*Xiphias gladius*	100 100	AB470301 *HM007787*
	1	**Filleted**	**COI**	***Ruvettus pretiosus*** **(oilfish)**	**99.9**	***Ruvettus pretiosus*** **(oilfish)**	**99** **99**	**EU752173** ***HM007721***
Tomato rockcod (*Cephalopholis sonnerati*)	1	Whole	COI	*Cephalopholis sonnerati*	99.8	*Cephalopholis sonnerati*	99	*HQ611096*
Tuna chunks in brine (Not specified)	5	Canned	COI	Failed to amplify with COI cocktail				
Wahoo (*Acanthocybium solandri*)	**1**	**Filleted**	**COI**	***Scomberomorus commerson*** **(Spanish / king mackerel)**	**100**	***Scomberomorus commerson*** **(Spanish / king mackerel)**	**100** **100**	**DQ885055** ***HM007790***
White steenbras (*Lithognathus lithognathus*)	**1**	**Filleted**	**COI**	***Taractichthys longipinnis*** **(bigscale pomfret)**	**99.5**	***Taractichthys longipinnis*** **(bigscale pomfret)**	**99**	**EF609476**
	1	**Filleted**	**COI**	***Ruvettus pretiosus*** **(oilfish)**	**99.9**	***Ruvettus pretiosus*** **(oilfish)**	**99**	**EU752173**
White stumpnose (*Rhabdosargus globiceps*)	3	Whole	COI	*Rhabdosargus globiceps*	100	*Rhabdosargus globiceps*	100	*HM007759*
Yellowbelly rockcod (*Epinephelus marginatus*)	1	Whole	COI	*Epinephelus marginatus*	100	*Epinephelus marginatus*	100	*HQ611093*
Yellowfin tuna (*Thunnus albacares*)	3	Filleted	COI	*Thunnus albacares* *Thunnus atlanticus* *Thunnus obesus* *Thunnus tonggol*	100 99.9 99.9 99.7	*Thunnus albacares* *Thunnus atlanticus* *Thunnus obesus* *Thunnus tonggol*	100 99 99 99	EF609629 DQ107588 DQ107642 DQ107634
			CR	------------------	------	*Thunnus albacares*	99 99	AF301200 *HQ853215*
	1	**Filleted**	**COI**	***Katsuwonus pelamis*** **(skipjack tuna)**	**100**	***Katsuwonus pelamis*** **(skipjack tuna)**	**100**	**GU225630**
Yellowtail (*Seriola lalandi*)	4	Filleted	COI	*Seriola lalandi*	100	*Seriola lalandi*	99 100	EF609460 *HM007727*
	5	**Filleted**	**COI**	***Seriola quinqueradiata*** **(Japanese amberjack)**	**100**	***Seriola quinqueradiata*** **(Japanese amberjack)**	**100**	**HQ641665**

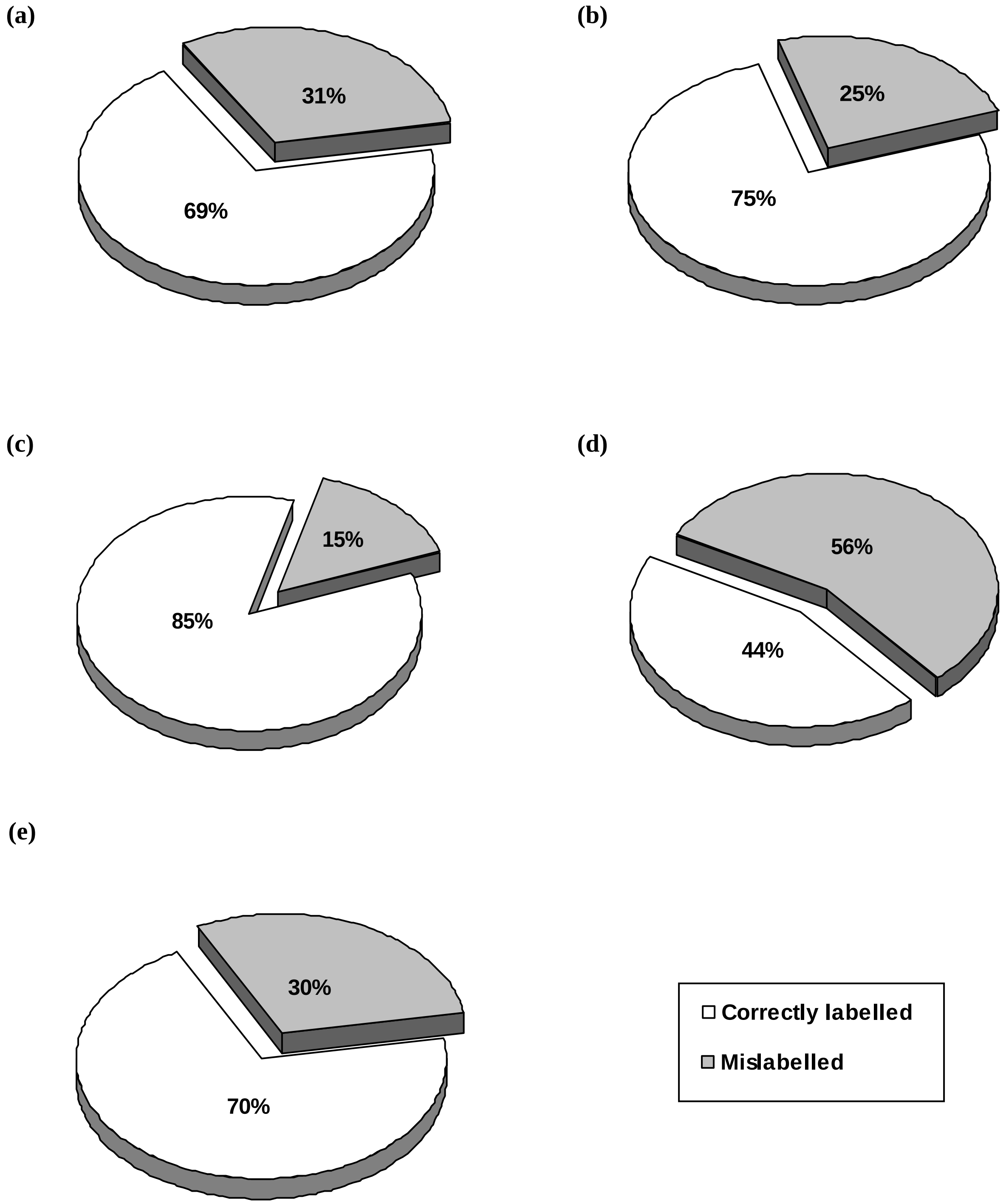


Figure 1 The percentages of fish samples obtained from retail outlets that were found to be mislabelled in (a) all four provinces surveyed (n = 140), (b) the Western Cape (n = 51), (c) the Eastern Cape (n = 27), (d) KwaZulu-Natal (n = 34), and (e) Gauteng (n = 28).

In South Africa, it has long been recognised that the popular and highly-valued kingklip (*Genypterus capensis*) is prone to market substitution (Smith & Smith, 1966). Kingklip stocks were heavily exploited in the 1980s by an experimental longline fishery in this country and these have not since recovered to their former abundance (McLean & Glazewski, 2009). Today, kingklip is managed as a bycatch in the offshore demersal trawl and demersal longline fisheries in South Africa, with a total allowable catch (TAC) of only about 3 000 tons per annum (compared to the TAC of *ca.* 150 000 ton per annum for hake) (DEAT, 2005; SASSI, 2010). In spite of this stringent limit, kingklip remains one of the most commonly marketed fish species on the domestic market (Cawthorn *et al*., 2011). While it is suspected that the congeneric counterpart from Australia or New Zealand known as ling/pink cusk eel (*Genypterus blacodes*) may serve as a substitute for kingklip (SASSI, 2010; Von der Heyden *et al*., 2010), this suspicion has not been definitively confirmed to date through DNA analysis. Although *G. capensis* and *G. blacodes* have been observed to be genetically closely-related, indistinguishable for example by 16S ribosomal RNA (rRNA) gene sequencing (Von der Heyden *et al*., 2010), it was shown in this study that the two species possess unique COI barcodes and can be readily discriminated on this basis. Indeed, 3 of the 12 (25%) retail samples marketed as ‘kingklip’ exhibited 100% sequence similarity with *G. blacodes* (Table 2), in spite of a number of COI sequences for *G. capensis* being available in the BOLD and GenBank databases. This form of mislabelling may not only defraud consumers financially, but it also likely creates a skewed impression of the abundance of kingklip stocks in South Africa, contradicting the messages being sent by organisations such as SASSI on the conservation status of the species.

Other cases of convoluted labelling observed at the retail level involved the use of generic or group names to describe certain fish samples. One sample labelled as ‘linefish’ was found to be geelbek/Cape salmon (*Atractoscion aequidens)* (Table 2), a species that has been heavily exploited by overfishing in the past (Hutton *et al*., 2001). Although *A. aequidens* is indeed a species caught in the traditional South African linefishery, there are approximately 150 other linefish species caught in this country (van Schalkwyk, 2007). These species may not only differ in commercial value, but they can also have markedly different conservation statuses. In addition, retail samples labelled by the generic term ‘red fish’ provided 100% sequence similarities with two endemic species of the Sparidae family, namely panga seabream (*Pterogymnus laniarius*) and slinger seabream (*C*. *puniceus*) (Table 2). Panga is among the most abundant of seabreams occurring in South African waters (Van der Elst, 1997), while stocks of slinger have been heavily exploited as a result of extensive fishing pressure (Chopelet *et al*., 2009). Therefore, this data emphasises that the ambiguities arising from the use of vernacular or generic names to group multiple species holds important ramifications for sustainable seafood campaigns, with the potential to prevent even the most knowledgeable of consumers from making informed purchases for the benefit of conservation.

Twenty-eight of the 42 (67%) incidents of retail-level mislabelling involved species which were from entirely different genera to the ones declared. Some of these cases appeared to have no clear explanation, with no apparent association with any form of financial or marketing incentive. Examples of such cases, most of which were perpetuated in KZN, included samples labelled as 'wahoo' which were identified as king mackerel (*Scomberomorus commerson*) and, conversely, 'barracuda' that turned out to be wahoo (*Acanthocybium solandri*) (Table 2). Samples of 'Cape salmon' and 'sailfish' were found to be substituted with shortbill spearfish (*T. angustirostris*), while big-scale pomfret (*Taractichthys longipinnis*) was misdescribed as 'angelfish'. Additionally, one sample denoted as 'codfish' on the label showed 99% sequence similarity with angelfish (*Brama brama*). When questioning the vendor on the origin of the 'cod' sample, it was indicated that the fish was caught in the local offshore trawl. Ironically, while cod (*Gadus* spp.) is heavily exploited in many parts of the world, it is not caught anywhere close to South Africa (Froese & Pauly, 2011). Apart from the possibility that the incorrect names were used to increase the market appeal of these samples, it appeared more likely in some instances that the misrepresentation was simply a result of ignorance on the part of the retailers. Confused nomenclature could also have been a reason for the mislabelling of kingklip as 'kingfish', where the retailer may not have recognised that these refer to different species (Table 2). The marketing of kingfish, nevertheless, presents an obstacle to consumer education and conservation strategies. All 53 species of kingfish are designated specially-protected and are illegal to sell in South Africa. However, these legal provisions would likely go unnoticed by the public if these species are touted on the market. In KZN, *Scomberomorus plurilineatus* is normally referred to as 'Natal snoek', but by shortening the name to just 'snoek', the vendors may not have comprehended that they were referring to a different species (*Thyrsites atun*). Furthermore, snoek (*T. atun*) may often be sold under the name 'barracouta', particularly when the species is imported. The one sample sold as 'barracuda' (expected to be *Sphyraena* spp.) which was found to be 100% similar to *T.*

atun (Table 2), may have potentially represented an instance where the mere misspelling of the market name altered the species being referred to. Even in the case that the aforementioned mislabelling incidents were unintentional, these results highlight the need for improved and more uniform fish naming in South Africa as a means of promoting fair trade, conservation efforts and consumer rights.

On the other hand, a number of cases of potentially deliberate mislabelling were uncovered, where financial incentives could have been a driving factor for the substitution of highly-valued fish species with lower-valued ones. One sample denoted as the highly-priced ‘Norwegian salmon’ on the product label showed 100% sequence similarity with the cheaper member of the Salmonidae family, rainbow trout (*Oncorhynchus* mykiss) (Table 2). Another sample labelled as ‘halfmoon rockcod’ turned out to be the totally unrelated and lower-valued jacopever (*Helicolenus dactylopterus*). In addition, COI sequencing showed that a sample marketed as ‘yellowfin tuna’ was actually more genetically similar to the less-valuable skipjack tuna (*Katsuwonus pelamis*). Probably the most prominent case of economic fraud at the retail level concerned the misrepresentation of five fish samples sold as ‘red snapper’ (Table 2). According to the US FDA list of acceptable market names for fish (FDA, 2010), the term ‘red snapper’ is the legally designated common name for *Lutjanus campechanus*, a highly-prized but severely overexploited species found in the southern Atlantic and Gulf of Mexico (Jacquet & Pauly, 2008; Froese & Pauly, 2011). However, the results of this study showed that a variety of alternative species may masquerade under this title to increase their market appeal, a finding consistent with numerous reports of ‘snapper’ mislabelling from other parts of the world (Hsieh *et al*., 1995; Marko *et al*., 2004; Logan *et al*., 2008; Wong & Hanner, 2008). COI sequencing revealed that two samples marketed as ‘red snapper’ exhibited 100% sequence similarity with other red-coloured species, namely panga seabream (*Pterogymnus laniarius*) and Roman seabream (*C. laticeps*) (Table 2). In addition, the discovery of three samples of river snapper (*Lutjanus argentimaculatus*) incorrectly labelled as ‘red snapper’ has serious conservation and legal ramifications. Since river snapper is an estuarine-dependant species with a high vulnerability to overexploitation, it has been designated as a recreational species only and is prohibited to sell in South Africa. However, when river snapper is concealed under the name ‘red snapper’, consumers are unable to avoid purchasing an illegal, locally-protected species.

Also of concern from a legality and conservation perspective was the blatant marketing of a number of samples in KZN retail outlets as ‘musselcracker’ and ‘white steenbras’/‘pignose grunter’. The term ‘musselcracker’ may refer to either one of the severely exploited black musselcracker (*Cymatoceps nasutus*) or white musselcracker (*Sparodon durbanensis*), with the latter being a recreational species prohibited for sale in South Africa. On the other hand, the market names ‘white steenbras’ and ‘pignose grunter’ both refer to the illegal-to-sell species *Lithognathus lithognathus*, the stocks of which are considered collapsed due to extensive overfishing (Bennett, 1993; SASSI, 2010). In spite of the use of these terms, two samples denoted as ‘musselcracker’ showed a 100% sequence similarity in BOLD with *Pseudopentaceros richardsoni* (pelagic armourhead), a species of limited commercial value in South Africa. A further sample sold as ‘musselcracker’ in the WC exhibited a 100% sequence similarity with bluenose warehou (*Hyperoglyphe antarctica*). This probably represented a case of intentional substitution as the latter is normally imported (Von der Heyden *et al*., 2010), while the former species are endemic to South Africa (SASSI, 2010). Similarly, ‘white steenbras’/‘pignose grunter’ was found to be misrepresented by a number of different species, including big-scale pomfret (*T. longipinnis*) and yellowtail (*S. lalandi*). At a first glance, the substitution of overexploited fish species with more sustainable ones may not be considered unfavourable from a conservation viewpoint. Nonetheless, this remains a serious form of deception and exemplifies a common problem faced by seafood awareness campaigns in their endeavors to educate consumers and provide tools for informed purchasing decisions. Similar to the repercussions discussed in terms of the mislabelling of kingklip, the selling of protected or illegal species (even if they are not what they are indicated to be) conceals the true status of the stocks. Consumers would probably be led to believe that the heavily exploited musselcrackers and white steenbras are actually plentiful if they are perceived to be continually available in the marketplace.

Lastly, cases of species mislabelling warranting concern from a health perspective involved the substitution of samples labelled as ‘swordfish’ and ‘white steenbras’ with oilfish (*Ruvettus pretiosus*). It is well established that oilfish contain high levels of indigestible wax esters, the purgative effects of which have been implicated in numerous cases of oily diarrhoea (keriorrhea) worldwide (Ling *et al*., 2008). Although the sale and import of oilfish has been banned in Italy, Japan and South Korea (Ling *et al*., 2009), oilfish is still sold in many countries due to its frequent bycatch in swordfish and tuna fisheries (Tserpes *et al*., 2006). The sale of oilfish is not prohibited in South Africa and no guidelines exist for the marketing of this fish. The fact remains that even if individuals wished to avoid the consumption of a species which could knowingly cause illness, they are denied the right to do so when it is labelled as 'swordfish' or 'steenbras'.

Regulatory aspects and mislabelling

Although many potential reasons for mislabelling have been presented here, these all ultimately stem from a lack of control on the fisheries market in South Africa. This lack of control can inevitably be traced back to the prevailing government legislation, where the policies relating to the marketing of fishery products are clearly inadequate and/or poorly enforced. The existing regulations in South Africa relating to the labelling of packaged foods have been published by the Department of Health (DoH), while those pertaining to the labelling of local and imported frozen fish products fall under the domain of the National Regulator for Compulsory Specifications (NRCS). Both of these sets of regulations specify that product labelling should not be misleading to the consumer (NRCS, 2003; DoH, 2010). In addition, the NRCS regulations expressly stipulate that a 'true description (of the) variety of fish' must be stated on the packaging of frozen fish products. In spite of these requirements, neither of these regulations include any rules or guidelines on the acceptable market names that should be used for different imported and domestic fish sold in South Africa, nor do they require the declaration of the Latin names for the species being marketed. Such weaknesses in the legal provisions evidently provide seafood suppliers with a substantial amount of leeway in terms of the names they use to sell their products, providing ample opportunities for mislabelling and fraud to present itself on the local market.

Confusion in fish nomenclature has been one of the driving factors for the compilation of lists of 'acceptable market names' for seafood products by many countries, such as Canada's Food Inspection Agency Fish List, the FDA's seafood list in the US and those lists compiled by member states of the European Union (EU) (BIM, 2001; CFIA, 2010; FDA, 2010; FSA, 2010). The establishment of such lists in South Africa could provide some relief to the problems of mislabelling observed in this study, however, it is unlikely that these alone will completely eliminate the problem. A number of studies have shown that seafood mislabelling persists in both the US and EU in spite of their possession of uniform seafood naming lists, where poor enforcement, irregular updating and a lack of harmonisation between trading partners have been cited as factors impeding the usefulness of such endeavors (Marko *et al*., 2004; Logan *et al*., 2008; Wong & Hanner, 2008; Miller & Mariani, 2010). The ambiguities existing in market nomenclature in today's global economy plainly signal the need for the additional declaration of the Latin names of fish species on product labels (Gerson *et al*., 2008). This practice has been made mandatory in the EU (EC, 2000; 2001) and, in the context of the results obtained here, is one that could markedly improve transparency and advance uniformity in the labelling of fish species in South Africa. In addition, the implementation of traceability policies for fishery products in this country, as have been implemented under EU law (EC, 2002), could promote industry responsibility, assisting in the curtailing of fraud and the prevention of the leakage of illegal, unreported, and unregulated (IUU) fishing products into the market (FAO, 2001; Miller & Mariani, 2010).

Exacerbating the complexities faced in this country due to the inadequacy of regulations are those problems associated with the monitoring and enforcement of the existing ones. At present, regulatory authorities inspect only a small portion of the fish products traded on the local marketplace to verify that the species are correctly identified. It is questionable as to whether the authentication methods presently utilised by the regulators, which rely on sensory evaluation to detect fish substitutions (P Trutter, 2010, NRCS, South Africa, personal communication), are sufficiently accurate for the purposes intended. The use of more sophisticated and validated species authentication techniques, such as the COI barcoding method used in this study, would certainly enhance the reliability of regulatory monitoring in South Africa, whilst providing superior evidence to warrant the prosecution of illegal activities.

Lastly, since regulatory bodies cannot be expected to inspect or test every fish product that appears on the market, the whole fisheries supply chain will undoubtedly need to take more responsibility in ensuring that the labelling of the fish products they sell is sufficiently informative and truthful. The latter will become increasingly important in the light of the recently published Consumer Protection Act (DTI, 2009) in South Africa, the regulations of which came into effect in April 2011. This Act aims to protect consumers from exploitation of any kind. Since the entire supply chain will be held responsible for any incident or complaint relating to their products, the individual links of the chain will be required to prove their credibility in order to avoid considerable penalties.

Conclusions

Consumers should be able to expect that the information presented on fish products is correct, especially at a time when they are increasingly being encouraged to take responsibly for both their own health and the health of the environment. This study represents the first comprehensive report on the use of DNA barcoding to investigate the prevalence of fish species substitution and fraud on the South African market. The results presented here have highlighted that the mislabelling of fish species is a reality in this country, being particularly pronounced at the retail level and in terms of processed fish products. The lax application of generic group names or the use of more exotic names to increase the market appeal of fisheries products is not only potentially impacting consumers financially, but it may also be jeopardising their health. Of paralleled concern is that the prevailing frequency of species substitutions is likely to be contradicting the messages on conservation being sent by organisations such as SASSI, while undoubtedly hampering the ability of proactive consumers to make sustainable seafood choices.

Clearly, such findings raise considerable concern on the functioning of the fisheries supply chain in South Africa and should compel authorities to identify targets for improving labelling policies, applicable to both domestic and imported products. Modifications to the existing legislation should, at the very least, include the requirement for the declaration on product labels of a designated ‘acceptable market name’ and Latin name of the fish species being traded. Obviously, such regulations will be of little value if they are not properly enforced. Government will consequently also need to address questions on whether the current regulatory monitoring activities are adequate and if the penalties imposed for non-compliance are sufficient to dissuade dishonest practices. Against this backdrop, DNA barcoding had been confirmed as an extremely powerful and widely applicable technique for fish identification purposes. The utilisation of such a method could offer a superior level of precision to fish authentication monitoring by both regulators and the industry, whilst also providing an important tool to justify the issuing of penalties and the prosecution of unlawful practices. If greater transparency can be achieved on the market through regulator and industry co-operation, then public confidence might be restored in the seafood supply chain in South Africa and full efforts may be refocused on the conservation of the ocean’s fish stocks, which are evidently not as ‘inexhaustible’ as once thought.

References

Aboim, M.A., Menezes, G.M., Schlitt, T. & Rogers, D. (2005). Genetic structure and history of populations of the deep-sea fish *Helicolenus dactylopterus* (Delaroche, 1809) inferred from mtDNA sequence analysis. *Molecular Ecology*, **14**, 1343-1354.

Alvarado Bremer, J.R. (1994). Assessment of morphological and genetic variation of the swordfish (*Xiphias gladius* Linnaeus): evolutionary patterns of nucleotide substitution in the mitochondrial genome. PhD Thesis, University of Toronto, Canada (As cited by Viñas & Tudela, 2009).

Alvarado Bremer, J.R., Naseri, I. & Ely, B. (1997). Orthodox and unorthodox phylogenetic relationships among tunas revealed by the nucleotide sequence analysis of the mitochondrial control region. *Journal of Fish Biology*, **50**, 540-554.

Atkins, H. (2010). You could be eating shark meat and not even know it. *Cape Times*, May 14, 2010.

Barbuto, M., Galimberti, A., Ferri, E., Labra, M., Malandra, R., Galli, P. & Casiraghi, M. (2010). DNA barcoding reveals fraudulent substitutions in shark seafood products: the Italian case of ‘‘palombo” (*Mustelus* spp.). *Food Research International*, **43**, 376-381.

Beddington, J.R., Agnew, D.J. & Clark, C.W. (2007). Current problems in the management of marine fisheries. *Science*, **316**, 1713-1716.

Bennett, B.A. (1993). The fishery for white steenbras *Lithognathus lithognathus* off the Cape coast, South Africa, with some considerations for its management. *South African Journal of Marine Science*, **13**, 1-14.

BIM (Bord Iascaigh Mhara) (2001). Information note on the labelling of fishery and aquaculture products according to Council Regulation (EC) No 104/2000 and Commission Regulation (EC) No 2065/2001. Dublin, Ireland: BIM.

Brown, J.R., Bechenbach, A.T. & Smith, M.J. (1993). Intraspecific DNA sequence variation of the mitochondrial control region of white sturgeon (*Acipenser transmontanus*). *Molecular Biology Evolution*, **10**, 326-341.

Brunner, E., Jones, P.J.S., Friel, S. & Bartley, M. (2009). Fish, human health and marine ecosystem health: policies in collision. *International Journal of Epidemiology*, **38**, 93-100.

Buck, E.H. (2009). Seafood marketing: combating fraud and deception. [Internet document]. URL www.nationalaglawcenter.org/assets/crs/RL34124.pdf. 20 February 2011.

Carlsson, J., McDowell, J.R., Díaz-Jaimes, P., Carlsson, J.E.L., Boles, S.B., Gold, J.R. & Graves, J.E. (2004). Microsatellite and mitochondrial DNA analyses of Atlantic bluefin tuna (*Thunnus thynnus thynnus*) population structure in the Mediterranean Sea. *Molecular Ecology*, **13**, 3345–3356.

Cawthorn, D.M., Steinman, H.A. & Witthuhn, R.C. (2011). Evaluating the availability of fish species on the South African market and the factors undermining sustainability and consumer choice. *Food Control*, **22**, 1748-1759.

Cawthorn, D.M., Steinman, H.A. & Witthuhn, R.C. (2011). Establishment of a mitochondrial DNA sequence database for the identification of fish species commercially available in South Africa. *Molecular Ecology Resources*, **11**, 979-991.

CFIA (Canadian Food Inspection Agency) (2010). CFIA Fish List. [Internet document] URL www.inspection.gc.ca/english/fssa/fispoi/product/comnome.shtml. 28 June 2011.

Chauhan, T. & Rajiv, K. (2010). Molecular markers and their applications in fisheries and aquaculture. *Advances in Bioscience and Biotechnology*, **1**, 281-291.

Chopelet, J., Helyar, S., Mann, B. & Mariani, S. (2009). Novel polymorphic microsatellite loci for the protogynous hermaphrodite slinger sea bream (*Chrysoblephus puniceus*, Sparidae). *Molecular Ecology Resources*, **9**, 1223-1226.

Chow, S., Okamoto, H., Uozumi, Y., Takeuchi, Y. & Takeyama, H. (1997). Genetic stock structure of the swordfish (*Xiphias gladius*) inferred by PCR-RFLP analysis of the mitochondrial DNA control region. *Marine Biology*, **127**, 359-367.

Cohen, N.J., Deeds, J.R., Wong, E.S., Hanner, R.H., Yancy, H.F., White, K.D., Thompson, T.M., Wahl, M., Pham, T.D., Guichard, F.M., Huh, I., Austin, C., Dizikes, G. & Gerber, S.I. (2009). Public health response to puffer fish (tetrodotoxin) poisoning from mislabeled product. *Journal of Food Protection*, **72**, 810-817.

Dawnay, N., Ogden, R., McEwing, R., Carvalho, G.R. & Thorpe, R.S. (2007). Validation of the barcoding gene COI for use in forensic genetic species identification. *Forensic Science International*, **173**, 1-6.

DEAT (Department of Environmental Affairs and Tourism) (2005). Policy for the allocation and management of commercial fishing rights in the hake longline fishery. [Internet document]. URL www.feike.co.za/web/info/Hake%20Longline%-20Policy.doc. 14 July 2010.

Delgado, C.L., Wada, N., Rosegrant, M.W., Meijer, S. & Ahmed, M. (2003). *The Future of Fish: Issues and Trends to 2020*. Pp. 1-5. Washington DC, USA: International Food Policy Research Institute and World Fish Center.

DoH (Department of Health) (2010). Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act 54 of 1972), regulations relating to the labelling and advertising of foodstuffs (R. 146/2010). *South African Government Gazette*, **32975**, 3-53.

Donaldson, K.A. & Wilson, R.R. (1999). Amphi-panamic geminates of Snook (Percoidei: Centropomidae) provide a calibration of the divergence rate in the mitochondrial DNA control region of fishes. *Molecular Phylogenetics and Evolution*, **13**, 208-213.

DTI (Department of Trade and Industry) (2009). Consumer Protection Act. Act no. 68 of 2008. R. 467/2009. *Government Gazette*, **32186**, 1-186.

EC (European Commission) (2000). European Council Regulation No 104/2000 of 17 December 1999 on the common organization of the markets in fishery and aquaculture products. *Official Journal of the European Communities*, **L17**, 22-52.

EC (European Commission) (2001). Commission Regulation (EC) No 2065/2001 of 22 October 2001 laying down detailed rules for the application of Council Regulation (EC) No 104/2000 as regards informing consumers about fishery and aquaculture products. *Official Journal of the European Communities*, **L278,** 6-8.

EC (European Commission) (2002). Regulation (EC) No 178/2002 of the European Parliament and of the council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. *Official Journal of the European Communities*, **L31**, 1-24.

Faber, J.E. & Stepien, C.A. (1997). The utility of mitochondrial sequences for analyzing phylogenetic relationships among populations, species, and genera of the Percidae. In T.D. Kocher & C.A. Stepien (Eds.), *Molecular systematics of fishes*, pp. 129-143. London: Academic Press.

FAO (Food and Agriculture Organization of the United Nations) (2001). International plan of action to prevent, deter and eliminate illegal, unreported and unregulated fishing. [Internet document]. URL www.fao.org/DOCREP/003/y1224e/y1224e00.-htm. 12 March 2011.

FAO (Food and Agriculture Organization of the United Nations) (2009). *The State of World Fisheries and Aquaculture 2008*. Pp. 1-175. Rome, Italy: FAO.

FDA (Food and Drug Administration) (2010). The seafood list - FDA's guide to acceptable market names for seafood sold in interstate commerce. [Internet document]. URL www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/Seafood/ucm113260.htm. 21 March 2011.

Filonzi, L., Chiesa, S., Vaghi, M. & Nonnis Marzano, F. (2010). Molecular barcoding reveals mislabelling of commercial fish products in Italy. *Food Research International*, **43**, 1383-1388.

Froese, R. & Pauly, D. (2011). FishBase: World Wide Web electronic publication, version 02/2011. [Internet document]. URL www.fishbase.org. 21 March 2011.

FSA (Food Standards Agency) (2010). Table of commercial designations for specific fish species derived from the Fish Labelling (England) Regulations 2010. [Internet document]. URL www.food.gov.uk/multimedia/pdfs/fishcomdesig.pdf. 21 November 2010.

Gerson, H., Cudmore, B., Mandrak, N.E., Coote, L.D., Farr, K. & Baillergeon, G. (2008). Monitoring international wildlife trade with coded species data. *Conservation Biology*, **22**, 4-7.

Hall, T.A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95-98.

Hajibabaei, M., Smith, M.A., Janzen, D.H., Rodriguez, J.J., Whitfield, J.B. & Hebert, P.D.N. (2006). A minimalist barcode can identify a specimen whose DNA is degraded. *Molecular Ecology Notes*, **6**, 959-964.

Handy, S.M., Deeds, J.R., Ivanova, N.V., Hebert, P.D.N., Hanner, R.H., Ormos, A., Weigt, L.A., Moore, M.M. & Yancy, H.F. (2011). A single-laboratory validated method for the generation of DNA barcodes for the identification of fish for regulatory compliance. *Journal of AOAC International*, **94**, 201-210.

Hebert, P.D.N., Cywinska, A., Ball, S.L., & deWaard, J.R. (2003a). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B*, **270**, 313-321.

Hebert, P.D.N, Ratnasingham, S. & deWaard, J.R. (2003b). Barcoding animal life: cytochrome *c* oxidase subunit I divergences among closely related species. *Proceedings of the Royal Society B*, **270**, S96-S99.

Hsieh, Y., Woodward, B.B. & Blanco, A.W. (1995). Species substitution of retail snapper fillets. *Journal of Food Quality*, **18**, 131-140.

Hutton, T., Griffiths, M.H., Sumaila, U.R. & Pitcher, T.J. (2001). Cooperative versus non-cooperative management of shared linefish stocks in South Africa: an assessment of alternative management strategies for geelbek (*Atractoscion aequidens*). *Fisheries Research*, **51**, 53-68.

Huxley, L. (2007). *The Life and Letters of Thomas Henry Huxley*. 296 p. Middlesex, UK: The Echo Library.

INFOSA (2007). Fisheries in the SADC region: selected country profiles. [Internet document]. URL www.infosa.org.na/dloads/restrict/country%20profile/Fisheries.pdf. 23 September 2010.

IUCN (International Union for Conservation of Nature) (2010). IUCN Red List of Threatened Species. Version 2010.2. [Internet document]. URL www.iucnredlist.org. 29 February 2011.

Ivanova, N.V., Zemlak, T.S., Hanner, R. & Hebert, P.D.N. (2007). Universal primer cocktails for fish DNA barcoding. *Molecular Ecology Notes*, **7**, 544-548.

Jacquet, J.L. & Pauly, D. (2007). The rise of seafood awareness campaigns in an era of collapsing fisheries. *Marine Policy*, **31**, 308-313.

Jacquet, J.L. & Pauly, D. (2008). Trade secrets: renaming and mislabelling of seafood. *Marine Policy*, **32**, 309-318.

Jie, H.A.N., Fenghua, L.V. & Houcai, C.A.I. (2011). Detection of species-specific long VNTRs in mitochondrial control region and their application to identifying sympatric Hong Kong grouper (*Epinephelus akaara*) and yellow grouper (*Epinephelus awoara*). *Molecular Ecology Resources*, **11**, 215-218.

Kaiser, M.J. & Edward-Jones, G. (2006). The role of ecolabeling in fisheries management and conservation. *Conservation Biology*, **20**, 393-398.

Lam, V. (2007). Oilfish - the case of the imitation Atlantic cod. *Sea Around Us Project Newsletter*, **40**, 1-2.

Lee, W.J., Conroy, J., Hunting Howell, W. & Kocher, T.D. (1995). Structure and evolution of teleost mitochondrial control region. *Journal of Molecular Evolution*, **41**, 54-66.

Ling, K.H., Cheung, C.W., Cheng, S.W., Cheng, L., Li, S.L., Nichols, P.D., Ward, R.D., Graham, A. & But, P.P.H. (2008). Rapid detection of oilfish and escolar in fish steaks: a tool to prevent keriorrhea episodes. *Food Chemistry*, **110**, 538-546.

Ling, K.H., Nichols, P.D. & But, P.P.H. (2009). Fish-induced keriorrhea. *Advances in Food and Nutrition Research*, **57**, 1-52.

Logan, C.A., Alter, S.E., Haupt, A.J., Tomalty, K. & Palumbi, S.R. (2008). An impediment to consumer choice: overfished species are sold as Pacific red snapper. *Biological Conservation*, **141**, 1591-1599.

Marko, P.B., Lee, S.C., Rice, A.M., Gramling, J.M., Fitzhenry, T.M., McAalister, J.S., Harper, G.R. & Moran, A.L. (2004). Mislabelling of a depleted reef fish. *Nature*, **430**, 309-310.

Martinez, I., James, D. & Loréal, H. (2005). Application of modern analytical techniques to ensure seafood safety and authenticity. *FAO Fisheries Technical Paper No. 455*. Pp. 1-73. Rome, Italy: FAO.

McLean, B. & Glazewski, J.I. (2009). Marine environments. In: *Fuggle & Rabie's Environmental Management in South Africa*, 2nd ed. (edited by H.A. Strydom & N.D. King). Pp. 455-511. Cape Town, South Africa: Juta.

Messing, J. (1983). New M13 vectors for cloning. *Methods in Enzymology*, **101**, 20-78.

Meusnier, I., Singer, G.A.C., Landry, J.F., Hickey, D.A., Hebert, P.D.N. & Hajibabaei, M. (2008). A universal DNA mini-barcode for biodiversity analysis. *BMC Genomics*, **9**, 214.

Miller, D.D. & Mariani, S. (2010). Smoke, mirrors, and mislabeled cod: poor transparency in the European seafood industry. *Frontiers in Ecology and the Environment*, **8**, 517-521.

NRCS (National Regulator for Compulsory Specifications) (2003). Compulsory Specification for Frozen fish, frozen marine molluscs and frozen products derived therefrom, VC 8017:2003, Ed. 3 (R. 979/2003). *South African Government Gazette*, **25172**, 3-50.

Ogden, R. (2008). Fisheries forensics: the use of DNA tools for improving compliance, traceability and enforcement in the fishing industry. *Fish and Fisheries*, **9**, 462-472.

Pardo, M.A. & Pérez-Villareal, B. (2004). Identification of commercial canned tuna species by restriction site analysis of mitochondrial DNA products obtained by nested primer PCR. *Food Chemistry*, **86**, 143-150.

Pauly, D., Watson, R. & Alder, J. (2005). Global trends in world fisheries: impacts on marine ecosystems and food security. *Philosophical Transactions of the Royal Society B*, **360**, 5-12.

Quinteiro, J., Sotelo, C.G., Rehbein, H., Pryde, S.E., Medina, I., Pérez-Martín, R.I., Rey-Méndez, M. & Mackie, I.M. (1998). Use of mtDNA direct polymerase chain reaction (PCR) sequencing and PCR-restriction fragment polymorphism methodologies in species identification of canned tuna. *Journal of Agricultural and Food Chemistry*, **46,** 1662-1669.

Ram, J.L., Ram, M.L. & Baidoun, F.F. (1996). Authentification of canned tuna and bonito sequence and restriction site analysis of polymerase chain reaction products of mitochondrial DNA. *Journal of Agricultural and Food Chemistry*, **44**, 2460-2467.

Rasmussen, R.S., Morrissey, M.T. & Hebert, P.D.N. (2009). DNA barcoding of commercially important salmon and trout species (*Oncorhynchus* and *Salmo*) from North America. *Journal of Agricultural and Food Chemistry*, **57**, 8379-8385.

Ratnasingham, S. & Hebert, P.D.N. (2007). The barcode of life database. *Molecular Ecology Notes*, **7**, 355-364.

Roheim, C.A. & Sutinen, J. (2006). Trade and marketplace measures to promote sustainable fishing practices. In: *International Trade and Sustainable Development Series Issue Paper No. 3*. Pp. 1-47. Geneva, Switzerland: International Centre for Trade and Sustainable Development and the High Seas Task Force.

Schlemmer, L. (1998). Gauteng: potential and challenges. In: *South Africa's magnifying glass: a profile of Gauteng province* (edited by P. Kok & R. Brandt). Pp. 23-35. Pretoria, South Africa: Human Sciences Research Council.

Smith, J.L.B. & Smith, M.M. (1966). *Fishes of the Tsitsikamma Coastal National Park*. P 102. Pretoria, South Africa: National Parks Board Trust.

Smith, J.L.B., Smith, M.M., & Heemstra, P.C. (2003). *Smiths' Sea Fishes*. P 1047. Cape Town, South Africa: Struik Publishers.

Smith, P.J., Struthers, C.D., Paulin, C.D., McVeagh, S.M. & Daley, R.K. (2009). Shallow genetic and morphological divergence among seaperches in the South Pacific (family Scorpaenidae; genus *Helicolenus*). *Journal of Fish Biology*, **74**, 1104-1128.

Steinke, D. & Hanner, R. (2011). The FISH-BOL collaborators protocol. *Mitochondrial DNA*, **22**, S10-S14.

Swartz, E.R., Mwale, M. & Hanner, R. (2008). A role for barcoding in the study of African fish diversity and conservation. *South African Journal of Science*, **104**, 293-298.

Thompson, M., Sylvia, G. & Morrissey, M.T. (2005). Seafood traceability in the United States: current trends, system design, and potential applications. *Comprehensive Reviews in Food Science and Food Safety*, **1**, 1-7.

Triantafyllidis, A., Karaiskou, N., Perez, J., Martinez, J.L., Roca, A., Lopez, B. & Garcia-Vazquez, E. (2010). Fish allergy risk derived from ambiguous vernacular fish names: forensic DNA-based detection in Greek markets. *Food Research International*, **43**, 2214-2216.

Trutter, P. (2010). National Regulator for Compulsory Specifications (NRCS), South Africa. Personal communication.

Tserpes, G., Tatamanidis, G. & Peristeraki, P. (2006). Oilfish and shark by-catches of the Greek swordfish fishery in the E. Mediterranean: a preliminary analysis applied to "presence-absence" data. *ICCAT Collective Volume of Scientific Papers*, **59**, 987-991.

Van der Elst, R. (1997). A guide to common sea fishes of Southern Africa. Pp. 7-22. South Africa: Struik Publishers.

Van Schalkwyk, M. (2007). Media release by the minister of environmental affairs and tourism: end of commercial fishing by middle of the century unless alternatives such as aquaculture are developed. [Internet document]. URL www.mcm-deat.gov.za/press/2007/10042007.html. 14 February 2011.

Viñas, J. & Tudela, S. (2009). A validated methodology for genetic identification of tuna species (genus *Thunnus*). *PLoS One*, **4**, e7606.

Von der Heyden, S., Barendse, J., Seebregts, A.J. & Matthee, C.A. (2010). Misleading the masses: detection of mislabelled and substituted frozen fish products in South Africa. *ICES Journal of Marine Science*, **67**, 176-185.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R. & Hebert, P.D.N. (2005). DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B,* **360**, 1847-1857.

Ward, R.D., Hanner, R. & Herbert, P.D.N. (2009). The campaign to DNA barcode all fishes, FISH-BOL. *Journal of Fish Biology*, **74**, 329-356.

Wong, H.K. & Hanner, R.H. (2008). DNA barcoding detects market substitution in North American seafood. *Food Research International*, **41**, 828-837.

Worm, B., Barbier, E.B. & Beaumont, N. (2006). Impacts of biodiversity loss on ocean ecosystem services. *Science*, **314**, 787-790.

Worm, B., Hilborn, R., Baum, J.K., Branch, T.A., Collie, J.S., Costello, C., Fogarty, M.J., Fulton, E.A., Hutchings, J.A., Jennings, S., Jensen, O.P., Lotze, H.K., Mace, P.M., McClanahan, T.R., Minto, C., Palumbi, S.R., Parma, A.M., Ricard, D., Rosenberg, A.A., Watson, R. & Zellar, D. (2009). Rebuilding global fisheries. *Science*, **325**, 578-585.

Yancy, H.F., Zemlak, T.S., Mason, J.A., Washington, J.D., Tenge, B.J., Nguyen, N.L., Barnett, J.D., Savary, W.E., Hill, W.E., Moore, M.M., Fry, F.S., Randolph, S.C., Rogers, P.L. & Hebert, P.D. (2008). The potential use of DNA barcodes in regulatory science: Applications of the Regulatory Fish Encyclopedia. Journal of Food Protection, **71**, 210-217.

Zhang, D.X. & Hewitt, G.M. (1996). Nuclear integrations: challenge for mitochondria DNA markers. *Trends in Ecology and Evolution*, **11,** 247-251.

Zhao, J.L., Wang, W.W., Li, S.F. & Cai, W.Q. (2006). Structure of the mitochondrial DNA control region of the Sinipercine fishes and their phylogenetic relationship. *Acta Genetica Sinica*, **33**, 793-799.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

Once viewed as the ultimate wilderness with an inexhaustible supply of fish, it has now become increasingly evident that the world's oceans have been seriously affected by human activities (Hyrenbach *et al*., 2000). Although many individuals undoubtedly comprehend the long-term consequences of overfishing, the drive to survive in the short term has led to the human race living according to the advice of Karl Marx, 'to each according to his needs' (Elster, 1986). The biologist Garrett Hardin likened the overfishing crisis to the 'tragedy of the commons' (Hardin, 1968). A resource that belongs to everyone but to no one, the ocean's fish stocks have been plundered by self-seeking individuals that have put their own privatised gains above the common loss in marine resources incurred by all individuals. As a result, today's fisheries have become largely unsustainable and grossly overcapitalised (Pauly *et al.*, 1998; Porter, 1998; Worm *et al.,* 2006). With too many boats chasing too few fish, and one trawler being capable of removing more than 60 000 tons of fish from the sea in a single haul, the depletion of numerous fish populations has been inevitable (Hillborn, 2007; Jacquet, 2009). As in many parts of the world, fish stocks in South Africa have also been overexploited due to excessive fishing, the results of which have not only caused damage to marine ecosystems, but have also had catastrophic consequences for many individuals who rely on fish as a source of food and income in this country (Nielsen & Hara, 2006; Sowman, 2006; Siebert, 2009).

James Madison stated in 1788 that 'if men were angels, no government would be necessary'. However, in a world in which resources are limited and man is far from angelic, a coercive means of controlling individual actions is often required (Kavka, 1995). Even although restrictions placed on fisheries (e.g. harvest quotas, area or seasonal closures and gear limitations) have become progressively more stringent, dishonest fishermen have responded by evading these stricter rules to compensate for their shrinking catches (Beddington *et al*., 2007; Brunner *et al*., 2009). Again, the self-seeking behaviour of man has perpetuated an enormous amount of illegal, unreported and unregulated (IUU) fishing, which, while enriching specific fisheries, has further eroded already vulnerable fish populations (Ogden, 2008; Agnew *et al.*, 2009). In addition, the human self-interest has carried through to global marketplaces, where marine resource scarcity has been masked by the fraudulent misnaming and mislabelling of fish species, either to conceal the fact that these are illegally caught or simply to achieve greater profits (Jacquet & Pauly, 2008).

Although illegal fishing and the mislabelling of fish has been suspected in South Africa for many years (Smith & Smith, 1966; Mann, 1995; SASSI, 2010), it has often been extremely difficult to confirm such cases due to the lack of accurate analytical methods available for identifying fish species in this country. This study aimed to highlight the problems existing in the trading of fish species on the South African market and to present solutions, in the form of the development of accurate molecular authentication methods, which could be applied at both an industry and regulatory level for the detection, deterrence and penalisation of illicit fishing and labelling practices.

The results obtained have shown that, in spite of the increased understanding of the state of decline of global and local fisheries, a large proportion of the fish being marketed in South African restaurants and retail outlets are those that are of conservation concern or even specially-protected and illegal-to-sell species (Chapter 3). Apart from this, the lack of accurate information on fish in the local marketplace is likely impeding the ability of South African consumers in making informed seafood purchasing decisions, whether these be based on sustainability or other considerations. The observed disparate manner in which fish species are named and the failure of seafood suppliers to comply with prevailing labelling regulations is likely creating a fisheries market in this country that is highly conducive to fraud and mislabelling. Such observations clearly indicate that the current regulations relating to the labelling of fish products in South Africa are either insufficient or are being poorly enforced, signaling the need for the revision of these regulations, the measures being applied to enforce them and the methods used to authenticate fish species in this country.

While DNA sequencing techniques are currently recognised as the most accurate means of making species identification (Unseld *et al*., 1995), such methods have not been extensively explored to date in South Africa for the identification of those fish species traded on the domestic market. In addition, reference DNA sequences for many locally-consumed fish species are not available in public databases, such as GenBank, for comparison with unknown sequences. This study aimed to fill this research void by evaluating different DNA extraction methods and DNA markers to establish the most suitable methods for the identification of fish species in South Africa. In a comparison of five different DNA extraction methods (Chapter 4), the SureFood® PREP kit was identified as the most applicable method for extracting high yields of pure DNA from fish muscle tissue. The ease-of use of this method, as well as its relative safety and suitability to high throughput applications, make this an ideal method for use in routine authentication testing of multiple fish species.

A comprehensive genetic database has been developed comprising DNA sequences from different mitochondrial loci of fish (16S ribosomal RNA (rRNA), 12S rRNA and cytochrome *c* oxidase I (COI) genes, as well as the control region), which should now permit the explicit identification of 53 commercial species traded in this country (Chapters 5 and 6). In a comparison of the three former gene regions, the COI gene has been shown to be the most appropriate DNA marker for fish authentication, allowing for the unambiguous identification of the vast majority (98%) of examined fish species. It is thus recommended that the COI region could serve as a universal genetic marker for routine authentication testing in South Africa, with only closely-related *Thunnus* species requiring further confirmation using mitochondrial control region sequencing and possibly also nuclear DNA analysis. Apart from allowing the identification of whole fish specimens, COI sequencing could also be used for the identification of fish at any developmental stage, including eggs, larvae, sibling and adult stages, as well as body fragments. The use of such methods would undoubtedly provide a greater level of precision to fish authentication testing by both the industry and regulatory bodies, not only enhancing transparency and fair trade on the domestic fisheries market, but also providing evidence for the prosecution of illegal activities and the issuing of penalties for non-compliance. In addition, from a fisheries research perspective, the value of integrating COI sequencing with classical identification approaches to validate existing taxonomic systems has been shown, as has the applicability of this method in revealing overlooked diversity and highlighting taxa which require additional investigation and resolution.

The utility of COI sequencing and the established genetic database has lastly been validated by testing the species authenticity of 248 fish samples collected from seafood wholesalers and retail outlets in South Africa (Chapter 7). The results emerging from this work have revealed that the misrepresentation of fish species is occurring deliberately or unintentionally on the South African market at various levels in the seafood supply chain and for various reasons. In total, 10 of 108 (9%) fish samples collected from local seafood wholesalers and 43 of 140 (31%) fish samples collected from retail outlets were identified as different species to the ones declared at the point of sale. While certain cases of the observed mislabelling were possibly due to ignorance or misapplied market nomenclature, many more of these appeared to be intentional acts of fraud for the purpose of accruing greater profits. Such mislabelling, therefore, does not only hold economic consequences, but certain substitutions were also detected that could have adverse effects on consumer health. In addition, consumers are almost certainly encountering imported fish species that are being substituted for local fish species, as well as locally-protected, illegal-to-sell species that are being sold under non-descript names, practices that undermine conservation efforts and potentially threaten overexploited fish stocks.

Concluding remarks and recommendations

Just as many individuals played a role in allowing the depletion of numerous fish populations around the world, the same individuals will ultimately need to play a role in rebuilding these stocks if man is to continue consuming and deriving an income from fish in the future. Improvement of the current overfishing crisis will thus require a shared responsibility on the parts of the fishing industry, fish suppliers, authorities, environmental organisations, as well as consumers. All of these parties need to be prepared to adopt and implement improved measures for sound seafood trade, with regular monitoring and auditing comprising a fundamental part of this process.

Unquestionably, fishermen can make one of the greatest contributions to the reversal of the overfishing problem by adhering to their harvest quotas and not overexploiting vulnerable species, but also by ensuring that they provide accurate information on the fish they catch to the next link in the seafood supply chain. Suppliers of fish in South Africa should consider more carefully the conservation status of the fish they are trading and the messages that they are conveying to consumers on the abundance of stocks when endangered or illegal species are marketed. In addition, fish purveyors need to be better trained on identifying the fish they have for sale and on providing more comprehensive information on the origin, production method and sustainability of these species. Packaged fish manufacturers need to abide more closely with labelling regulations and realise that their failure to provide crucial information on fish not only potentially damages the marine ecosystem, but may also decrease consumer confidence in their organisation and the entire fishing industry.

Authorities responsible for the regulation of fish products should recognise that misnaming and mislabelling is occurring on the market on a regular basis, and that the legislation in place to control these issues is either inadequate or is not being satisfactorily followed. In order to create uniformity in the marketplace and decrease the incidence of misnaming, it is recommended that authorities in South Africa consult with the fishing industry to generate and enforce a list of ‘acceptable market names’ for locally-traded species, including scientific and common names, as has been done in the United Kingdom, United States and Australia/New Zealand. Further to the declaration of an acceptable common name for the fish being sold, it is suggested that South Africa follow the example set by European regulatory systems and stipulate the mandatory declaration on product labels of the species name, geographical origin and production method (farmed or wild) of fish. The provision of such information will promote transparency in the fisheries market and assist consumers in distinguishing the quality, environmental impact and safety of the fish that they purchase.

Strict control and monitoring will be required on behalf of the appropriate authorities to ensure that the suggested regulations are adhered to by fish suppliers. As the problem of mislabelling is predominantly an economic issue, the response to the detection of fraudulent practices should also be dealt with in the economic realm, meaning that stricter penalties for non-compliance should be instituted. Since the sensory analyses currently used by South African regulators for species authentication appear to be inadequate, more advanced analytical methods to identify fish species are urgently required, particularly in the light of the increasing quantities of processed and foreign products on the market. DNA sequencing methods, particularly those based on the COI gene, have been shown to be highly effective for identifying fish at the species level. Together with the extensive database of reference DNA sequences for fish developed in this study, such methods could be readily and relatively economically applied for both industry self-regulation and for governmental monitoring.

Environmental organisations, such as the Southern African Sustainable Seafood Initiative (SASSI), need to continue educating consumers about the state of marine resources, providing advice on the most sustainable fish species to purchase and bridging a central gap between consumers and authorities. Finally, with increased confidence in the accuracy of the information that is being provided on fishery products at the point of sale, South African consumers will be in a better position to select fish that are known to be sustainable, and in so doing, decrease demand for those species which are not.

References

Agnew, D., Pearce, J., Pramod, G., Peatman, T., Watson, R., Beddington, J. & Pitcher, T. (2009). Estimating the worldwide extent of illegal fishing. *PLoS One*, **4**, e4570.

Beddington, J.R., Agnew, D.J. & Clark, C.W. (2007). Current problems in the management of marine fisheries. *Science*, **316**, 1713-1716.

Brunner, E., Jones, P.J.S., Friel, S. & Bartley, M. (2009). Fish, human health and marine ecosystem health: policies in collision. *International Journal of Epidemiology*, **38**, 93-100.

Elster, J. (1986). *An introduction to Karl Marx*. Pp. 96-97. Cambridge, UK: Cambridge University Press.

Hardin, G. (1968). The tragedy of the commons. *Science*, **162**, 1243-1248.

Hillborn, R. (2007). Managing fisheries is managing people: what has been learned? *Fish and Fisheries,* **8***,* 285-296*.*

Hyrenbach, K.D., Forney, K.A. & Daytin, P.K. (2000). Marine protected areas and ocean basin management. *Aquatic Conservation: Marine and Freshwater Ecosystems*, **10**, 437-458.

Jacquet, J.L. (2009). Fish as food in an age of globalization. PhD Thesis, University of British Columbia, Canada.

Jacquet, J.L. & Pauly, D. (2008). Trade secrets: renaming and mislabelling of seafood. *Marine Policy*, **32**, 309-318.

Kavka, G.S. (1995). Why even morally perfect people would need government. *Social Philosophy and Policy*, **12**, 1-18.

Nielsen, J.S. & Hara, M. (2006). Transformation of South African industrial fisheries. *Marine Policy*, **30**, 43-50.

Mann, B.Q. (1995). Quantification of illicit fish harvesting in the Lake St Lucia Game Reserve, South Africa. *Biological Conservation*, **74**, 107-113.

Ogden, R. (2008). Fisheries forensics: the use of DNA tools for improving compliance, traceability and enforcement in the fishing industry. *Fish and Fisheries*, **9**, 462-472.

Pauly, D., Christensen, V., Dalsgaard, J., Froese, R. & Torres, F. (1998). Fishing down marine food webs. *Science*, **279**, 860-863.

Porter, G. (1998). *Estimating Overcapacity in the Global Fishing Fleets*. P 20. Washington DC, USA: WWF.

SASSI (South African Sustainable Seafood Initiative) (2010). [Internet document]. URL www.wwfsassi.co.za. 14 July 2010.

Siebert, T. (2009). Food for thought. SANCOR (South African Network for Coastal and Oceanographic Research) Newsletter, Issue 188 of Jan/Feb 2009. [Internet document]. URL http://sancor.nrf.ac.za/newsletters/past-issues/issue-188/food-for-thought. 25 June 2010.

Smith, J.L.B. & Smith, M.M. (1966). *Fishes of the Tsitsikamma Coastal National Park*. P 102. Pretoria, South Africa: National Parks Board Trust.

Sowman, M. (2006). Subsistence and small-scale fisheries in South Africa: a ten-year review. *Marine Policy*, **30**, 60-73.

Unseld, M., Beyermann, B., Brandt, P. & Hiesel, R. (1995). Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. *PCR Methods and Applications*, **4**, 241-243.

Worm, B., Barbier, E.B. & Beaumont, N. (2006). Impacts of biodiversity loss on ocean ecosystem services. *Science*, **314**, 787-790.